DEVELOPMENT AND VALIDATION OF NEW ANALYTICAL METHODS FOR THE ESTIMATION OF RUFINAMIDE IN BULK AND PHARMACEUTICAL DOSAGE FORM

A Dissertation submitted to
THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
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In partial fulfilment of the requirements the award of the Degree of
MASTER OF PHARMACY
IN
BRANCH – III PHARMACEUTICAL ANALYSIS

Submited by
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Under the Guidance of
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DECLARATION

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EVALUATION CERTIFICATE

This is to certificate that the dissertation entitled **"DEVELOPMENT AND VALIDATION OF NEW ANALYTICAL METHODS FOR THE ESTIMATION OF RUFINAMIDE IN BULK AND PHARMACEUTICAL DOSAGE FORM"** submitted to The TamilnaduDr. M.G.R. Medical University, Chennai, is a bonafide project work of **Mr. V. RICARDO (Reg. No: 261731106)**, carried out in the Department of Pharmaceutical Analysis, Jaya College of Paramedical Sciences, College of Pharmacy, Thiruninravur, Chennai – 602 024, in partial fulfilment for the Degree of **MASTER OF PHARMACY** under the guidance of **Dr. C. ROOSEWELT, M.Pharm., Ph.D.,** Professor cum HOD, Department of Pharmaceutical Analysis, Jaya College of Paramedical Sciences, College of Pharmacy, Thiruninravur, Chennai – 602 024.

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LIST OF ABBREVIATIONS

AR	:	Analytical Reagent
API	:	Active Pharmaceutical ingredients
CV	:	Coefficient of Variation
Conc	:	Concentration
EMR	:	Electro magnetic radiation
gm	:	Gram
HBr	:	Hydrobromide
GLC	:	Gas Liquid Chromatography
HPLC	:	High Performance Liquid Chromatography
HPTLC	:	High Performance Thin Layer Chromatography
HPLC-MS	:	High Performance Liquid Chromatography and Mass Spectroscopy
ICH	:	International Conference on Harmonization
IUPAC	:	International Union of Pure and Applied Chemistry
LC	:	Liquid Chromatography
LC-MS	:	Liquid Chromatography and Mass Spectroscopy
LOD	:	Limit of Detection
LOQ	:	Limit of Quantitation
mcg	:	Micro gram
ml	:	Milli liter

mg	:	Milligram
nm	:	Nano meter
N	:	Theoritical plates
pg	:	Pico gram
R_f	:	Retardation Factor
Rs	:	Resolution
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
RP-TLC	:	Reverse Phase Thin Layer Chromatography
RSD	:	Relative Standard Deviation
R_t	:	Retention time
SD	:	Standard Deviation
TLC	:	Thin Layer Chromatography
T	:	Tailing factor
UV	:	Ultra- violet
USP	:	United States of Pharmacopoeia
v/v	:	Volume/Volume
WHO	:	World Health Organization
λ	:	Wavelength
λmin	:	Wavelength Minima
λmax	:	Wavelength Maxima

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1. INTRODUCTION

Analytical chemistry[1] is often described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively (what is present) and quantitatively (how much is present). Analytical chemistry is not a separate branch of chemistry, but simply the application of chemical knowledge.

Pharmaceutical Analysis[2] is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk and pharmaceutical preparation.

The technique[3] employed in quantitative analysis is based upon the quantitative performance of suitable chemical reactions and either measuring the amount of reagent needed to complete the reaction, or ascertaining the amount of reaction product obtained.

Quality[4] is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no “second quality” in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production.

Physico-chemical methods[5,6] are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the Physico-chemical methods, the most important are optical (Refractometry, Polarimetry, Emission, Fluorescence methods of analysis, Photometry including Photocolorimetry and Spectrophotometry covering UV-Visible and IR regions and Nephelometry or Turbidimetry) and chromatographic (Column, Paper, TLC, GLC, HPLC) methods.

Methods such as Nuclear Magnetic Resonance and Para Magnetic Resonance are becoming more and more popular. The combination of Mass Spectroscopy with Gas Chromatography and Liquid Chromatography are the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis.

The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of Pharmacopoeia.
3. The analysis should be economical.
4. The selected method should be precise and selective.

These requirements are met by the Physico-chemical methods of analysis, a merit of which is their universal nature that can be employed for analyzing organic compounds with a diverse structure. Of them, Visible Spectrophotometry is generally preferred especially by small scale industries as the cost of the equipment is less and the maintenance problems are minimal.

Instrumental methods of Chemical analysis:

Instrumental method is an exciting and fascinating part of chemical analysis that interacts with all areas of chemistry and with many other areas of pure and applied sciences. Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and environment.

This instrumentation provides lower detection limits required to assure safe foods, drugs, and water air. Instrumental methods are widely used by Analytical chemists to save time, to avoid chemical separation and to obtain increased accuracy.

Most instrumental techniques fit into one of the four principle areas. For convenience and better understanding introduction is divided into three parts,

A) Spectroscopy

B) Chromatography

C) Validation

Other chapters such as objectives, methodology, results, discussion and conclusion have been divided into two parts,

A) UV Spectrophotometry

B) High Performance Liquid Chromatography

PART-A -: SPECTROSCOPY[7]

Spectroscopy is the measurement and interpretation of Electro Magnetic Radiation (EMR) absorbed or emitted when the molecule or atoms or ions of a sample move from one energy state to another energy state. This change may be from ground state to excited state or excited state to ground state. At ground state, the energy of a molecule is the sum of rotational, vibrational and electronic energy. In other words, Spectroscopy measures the changes in rotational, vibrational and / or electronic energies.

Ultraviolet Spectroscopy:

Ultraviolet Spectroscopy is concerned with the study of absorption of UV radiation which ranges from 190 nm to 380 nm. Any molecule has n, π or σ or a combination of these electrons. These bonding (σ and π) and non bonding (n) electrons absorb the characteristic radiation and undergoes transition from ground state to excited state.

INSTRUMENTATION:

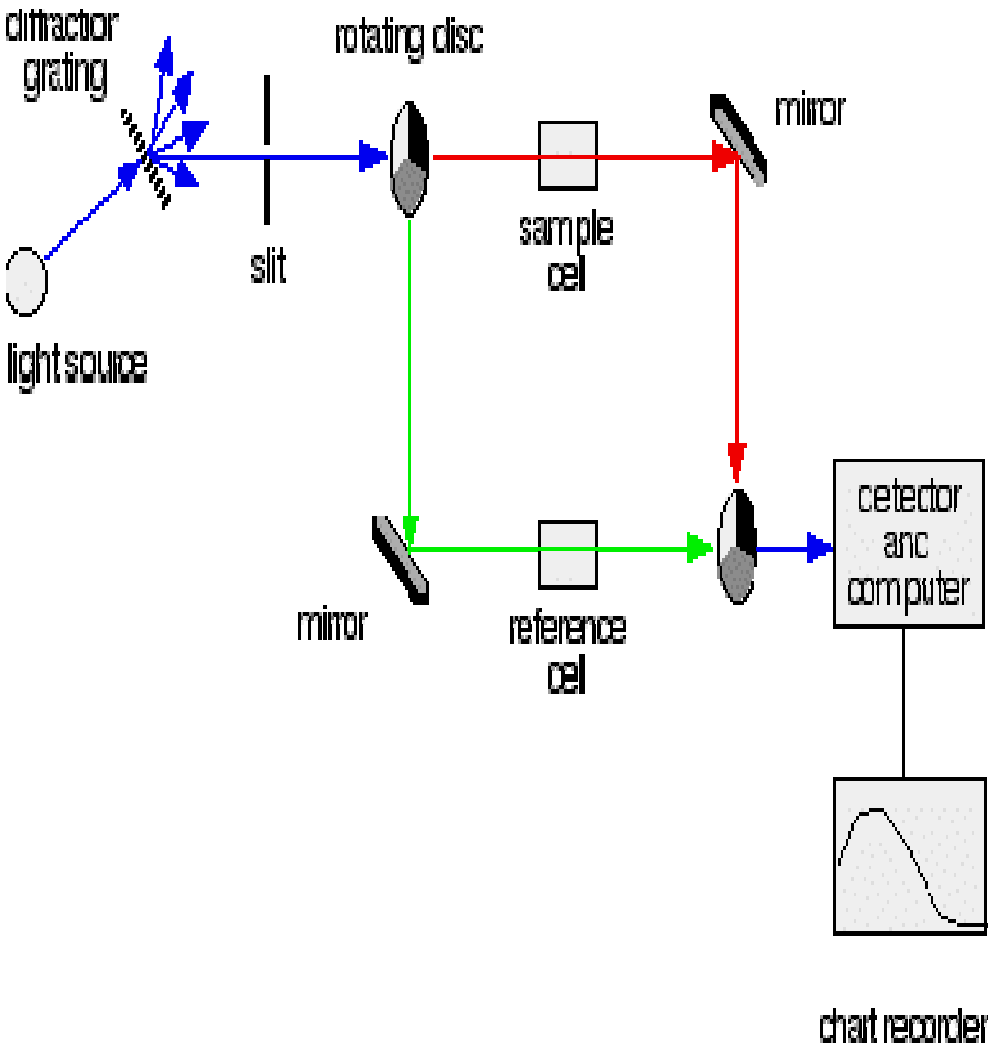


Fig: 1.1. UV Visible double beam Spectrophotometer

By the characteristic absorption peaks, the natures of the electrons present and hence the molecule structure can be elucidated. The component of double beam Spectrophotometer was shown in **Fig: 1.1.**

Visible Spectroscopy (Colorimetry):

Colorimetry is concerned with the study of absorption of visible radiation whose wavelength ranges from 380 nm to 780 nm. Any coloured substance will absorb radiation in this wavelength region. Coloured substances absorb light of different wavelength in different manner and hence we get an absorption curve (absorbance Vs wavelength). In this absorption curve, the wavelength at which maximum absorption of radiation takes place is called as λ_{max}. This λ_{max} is characteristic or unique for every coloured substance and this is a qualitative aspect, useful in identifying the substance. There are 4 types of transitions observed in UV visible spectroscopy, $\sigma \rightarrow \sigma^*$, $\pi \rightarrow \pi^*$, $n \rightarrow \sigma^*$, and $n \rightarrow \pi^*$.

AUC UV-SPECTROPHOTOMETRIC METHOD[10]:

The Area under Curve [AUC] method involves the calculation of integrated value of absorbance with respect to the wavelength between two selected wavelengths λ1 and λ2. Area calculation process calculates the area bound by the curve and the horizontal axis passes through the curve. The horizontal axis is selected by entering the wavelength range over which the area has to be calculated. This wavelength range was selected on the basis of repeated observations so as to get the linearity between AUC and concentration. AUC method has an advantage that when the drug shows spectra without sharp peak, it is possible to apply AUC method for determination of the drug with better accuracy and precision.

Terms used in absorption spectroscopy :

❖ **Transmittance (T):** It is the ratio of intensity of transmitted light to that of

incident light.

$$T = I_t / I_o$$

❖ **Absorbance (A):** It is the negative logarithm of transmittance to the base 10.

$$A = - \log_{10} T = \log_{10} I_o / I_t$$

$$A = abc$$

❖ **Molar absorptivity (ε):** When concentration „c in equation A= abc is expressed in mole/lit and cell length in „cm then Absorptivity is called as molar absorptivity.

$$\varepsilon = A / bc$$

❖ **Beer Lambert's law:** It can be stated that as the intensity of beam of monochromatic light when passed through transparent medium decreases exponentially as the thickness and concentration of absorbing media increases arithmetically.

$$A = \log I_o / I_t = abc$$

Where, A = Absorbance of the solution at particular wavelength of the light beam

I_o = Intensity of incident light beam

I_t = Intensity of transmitted light beam

a = Absorptivity of molecule at the wavelength of beam

b = Path length of cell in cm

c = Concentration of solution in gm/lit.

Beers law is said to be obeyed over a concentration range, if a plot of concentration against absorbance passes through origin and is a straight line and was shown in

Fig: 1.2.

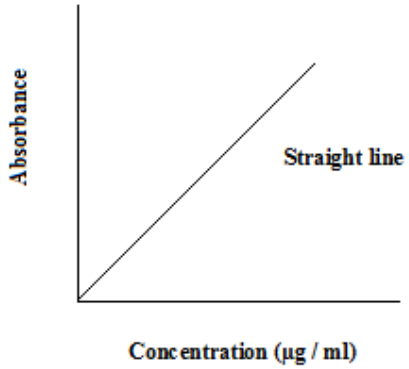


Fig: 1.2. Beer's law plot

Laws governing absorption of radiation:

Beer's law (related to concentration of absorbing species)

„The intensity of a beam of monochromatic light decreases exponentially with increase in the concentration of absorbing species arithmetically□.

Lambert's law (related to thickness / path length of absorbing species)

„The rate of decrease of intensity (monochromatic light) with the thickness of the medium is directly proportional to the intensity of incident light□.

***Limitations of Beer-Lambert's Law*:**

The Beer-Lambert law is rigorously obsessed provided a single species gives rise to the observed absorption. However the law may not obsessed when,

1. Different forms of the absorbing molecules are in equilibrium.
2. Solute and solvent from association complexes.
3. There is a thermal equilibrium between ground electronic state and a low lying excited state.
4. The compounds are charged by irradiation (fluorescent compounds).

Deviations from Beer's Law [2]**:**

According to Beer□s law, a straight line passing through the origin should be obtained,

when a graph is plotted between absorbance (A) and concentration (C). Deviation from the law may be positive or negative, according to whether the resulting curve is concave upwards or concave downwards. The deviations from the Beer□s law may be due to interaction of the solute molecules with each other or with the solvent or may be due to instrumental factors.

The most important reasons that cause deviations are

1. Negative deviation can always be expected when the illumination is not monochromatic.
2. The presence of impurities that fluoresce or absorb at the required absorption wavelength.
3. Environmental errors such as solvent, temperature and pressure.
4. Chemical factors such as change in pH and chemical equilibrium, presence of complexing agent, competitive metal ion reactions and concentration dependence.
5. Refractive index of sample.
6. Instrumental errors such as radiation, stability of radiation source, stability of slit control and electronics and reliability of the optical parts.

Choice of solvent [8]**:**

Several solvents used in Ultraviolet Spectroscopy with their cutoff wavelengths are listed in **Table: 1.1.**

A suitable solvent for UV Spectroscopy should meet the following requirements.

a. It should not absorb radiations in the region under investigation.
b. It should be less polar, so that it has minimum interaction with the solute molecules.
c. The solvent used should be of high purity.

Table: 1.1. List of common solvents used in UV Spectroscopy[4]

Solvents	Cut off wavelength (nm)
Acetonitrile	190
Water	191
Cyclohexane	195
Hexane	201
Methanol	203
Ethanol	204
Ether	215
Methylene dichloride	220
Chloroform	237
Dimethyl Sulfoxide	262

***Detectors*:**

A detector is a transducer that convert EMR into an electron flow and subsequently, into a current flow or voltage in the readout circuit. Photoelectric or Photo multiplier tubes are generally used as detectors. The detector must have the following important requirements. It must respond to radiant energy over a broad wavelength range.

a. It should be sensitive to low levels of radiant power.
b. It should rapidly respond to the radiation and produce an electrical signal that can be readily amplified.
c. It should have relatively low noise level (for stability).
d. The signal produced is directly proportional to the power of beam striking it.

Derivative Spectrophotometry[3, 9]:

Derivative Spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum and are shown in **Fig: 1.2.** In the context of Derivative Spectrophotometry, the normal absorption is referred to as fundamental, Zeroth order or D^0 spectrum.

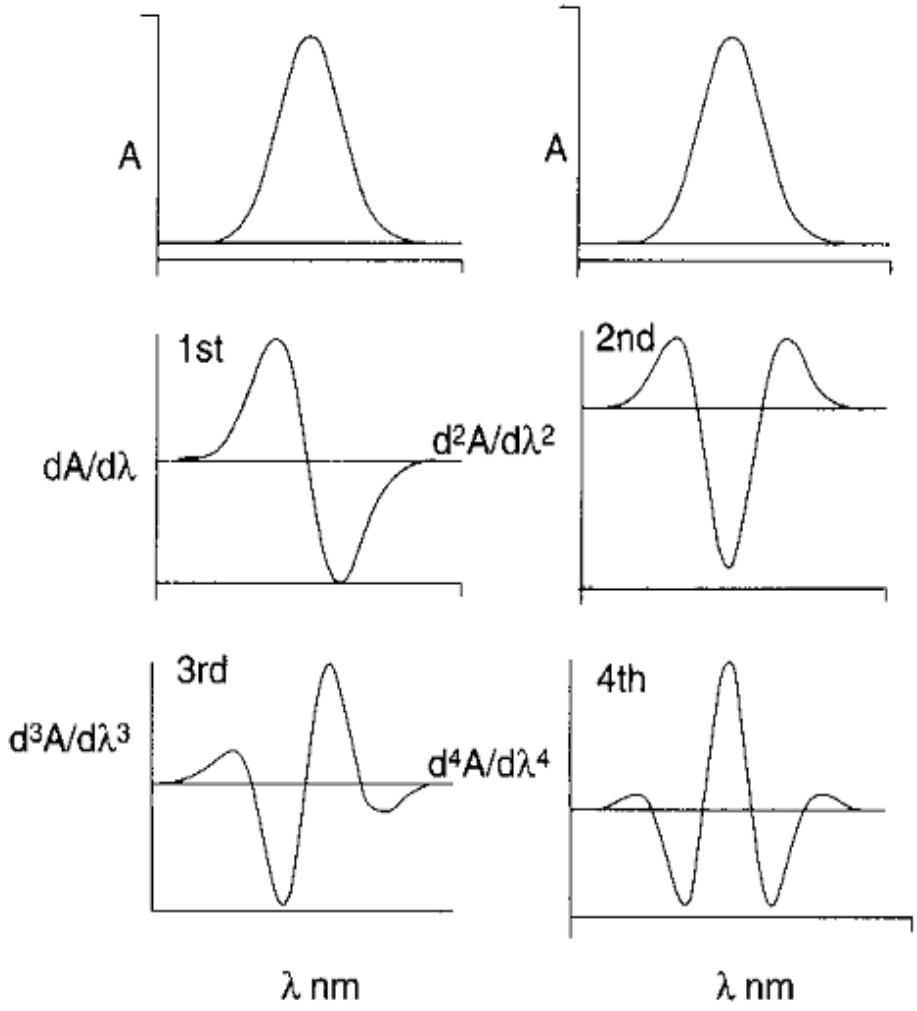


Fig: 1.3. Zero, First, Second, Third and Fourth order derivative spectra of Gaussian peak[10]

The First derivative spectrum (D^1) is a plot of the rate of change of absorbance with wavelength against wavelength, i.e. a plot of the slope of the fundamental spectrum

against wavelength or a plot of dA / dλ versus λ. The λ_{max} is a wavelength of zero slope and gives dA / dλ = 0, i.e. a cross-over point, in the D^1 spectrum.

The Second derivative spectrum (D^2) is a plot of the curvature of the D^0 spectrum against wavelength or a plot of $d^2A / d\lambda^2$ versus λ. The maximum negative curvature at λ_{max} in the D^0 spectrum gives a minimum in the D^2 spectrum.

In summary the First derivative spectrum of an absorption band is characterized by a maximum, a minimum, and a cross-over point at the λ_{max} of the absorption band. The Second derivative spectrum of an absorption band is characterized by two satellite maxima and an inverted band of which the minimum corresponds to the λ_{max} of the fundamental band.

The location of the end point of a potentiometric titration can often be accomplished more exactly from the First or Second derivative of the titration curve, than from the titration curve itself. Similarly, absorption observations will often yield more information from derivative plots than from the original absorption curve. This technique was used as long ago as 1955, but with the development of microcomputers which permit rapid generation of derivative curves, the method has acquired great impetus. If we consider an absorption band showing a normal (Gaussian) distribution, we find that the First and Third derivative plots are disperse functions that are unlike the original curve, but they can be used to fix accurately the wavelength of maximum absorption.

The Second and Fourth order derivatives have a central peak which is sharper than the original band but of the same height; its sign alternates with increasing order. It is clear that resolution is improved in the even-order spectra, and this offers the possibility of separating two absorption bands which may in fact merge in the Zero-order spectrum. Thus, a mixture of two substances gave a Zero-order spectrum

showing no well-defined absorption bands, but the second-order spectrum deduced from this curve showed well resolved peaks. The influence of an impurity on the absorption spectrum of a substance can often be eliminated by considering derivative curves.

The Second-order plot of the mixture is identical with that of pure substance. When the interference spectrum can be described by an n^{th}-order polynomial, the interference is eliminated in the (n + 1) derivative.

For quantitative measurements peak heights (expressed in mm) are usually measured of the long-wave peak satellite of either the second- or fourth-order derivative curves, or for the short-wave peak satellite of the same curves. Derivative spectra can be recorded by means of a wavelength modulation device in which beams of radiation differing in wavelength by a small amount (1-2 nm) fall alternately on the sample cell and the difference between the two readings is recorded. In an alternative procedure, a derivative unit involving resistance/capacitance circuits, filters and operational amplifier are attached to the Spectrophotometer, but as already indicated, derivative curves are most readily obtained by computer-based calculations.

Points to be considered before devising new analytical methods are:

Literature Survey:

Existing analytical methods for the compound to be analyzed are scanned to avoid duplication of the method. Further, the information about the solubility, absorbance maximas and the molar absorptivities in various solvents of the individual component formulation are obtained.

Selecting a solvent :

Solvent mixtures, in which all the components in the formulation are soluble and

stable, are chosen. Another point that needs consideration in selecting the solvent is difference in absorbance maximas of the component in the particular solvent. Greater the difference in the absorbance better will be the result.

Selecting the sampling wavelength:

Sampling wavelengths are selected considering the peaks and valleys in the UV Spectra of the individual component and the other wavelength where the various components show a difference in the absorbance.

Sample analysis and calculations:

The concentration of the sample solution is adjusted in such a way that the absorbance at the wavelength in the scanning region should be in the range of 0.5-1.5 Abs units. The analysis is repeated and accuracy, reproducibility is confirmed.

Type of instrument:

It is heart of the analytical method because more advanced the instrument greater will be the accuracy of the results and confidence with which the results are reported.

Evaluation of reproducibility:

To ensure that proper conditions have been selected and that no important variables have been overlooked, the tentative method should be critically evaluated with respect to Beer□s law.

STATISTICAL ANALYSIS[11]:

Statistical procedures and representative calculations:

The consistency and suitability of the developed method are substantiated through the statistical analysis like standard deviation, relative standard deviation and theoretical plates per meter.

For Accuracy:

- **Standard Deviation** = $\sigma = \sqrt{\frac{\sum (x - x_i)^2}{n-1}}$

Where, **x** = Sample,

$\mathbf{x_i}$ = Mean value of samples,

n = Number of samples

- **Relative Standard Deviation** = $\sigma / x_i \times 100$
- **Molar extinction coefficient (mol^{-1} cm^{-1})** = A / C × L

Where, A = Absorbance of drug

C = Concentration of drug

L = Path length

- **Sandell' s sensitivity (mcg / cm^2 / 0.001 absorbance units)** = C / A × 0.001

Where, C = Concentration of drug

A = Absorbance of drug

Unit- (mcg / cm^2 = 0.001 absorbance)

- **Coefficient of variance** (σ) = $\sum(x- \bar{x})^2$ / n-1
- **Regression equation** y = a + bx
- **Slope** = y / x

Where, x = Concentration

y = Absorbance

a = Intercept

- Limit of detection: (D_L) = 3.3 × σ / S

Units- (mcg / ml)

Where, σ = Standard deviation of the response.

S = Slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte.

The estimate of σ may be carried out in a variety of ways.

- Limit of quantitation (Q_L) = 10 × σ / S

Unit- (mcg / ml)

Where, σ = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimation of σ may be carried out in a variety of ways.

PART-B: CHROMATOGRAPHY[12]:

Chromatography, by classical definition, is a separation process where resolution is achieved by the distribution of the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed in the mobile phase. As a consequence solutes are eluted from the system in the order of their increasing distribution coefficients with respect to the stationary phase; ipso facto a separation is achieved. The mobile phase can be a gas or a liquid which gives rise to the two basic forms of Chromatography, namely, Gas Chromatography (GC) and Liquid Chromatography (LC). The stationary phase can also take two forms, solid and liquid, which provides two subgroups of GC and LC, namely; Gas-Solid Chromatography (GSC) and Gas-Liquid Chromatography (GLC), together with Liquid Solid Chromatography (LSC) and Liquid Liquid Chromatography (LLC).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1, 13, 14]:

In the modern pharmaceutical industry, HPLC is a major analytical tool applied at all

stages of drug discovery, development and production. Fast and effective development of rugged analytical HPLC methods is more efficiently undertaken with a thorough understanding of HPLC principles, theory and instrumentation.

Liquid Chromatography (LC), which is one of the forms of Chromatography, is an analytical technique that is used to separate a mixture in solution into its individual components. The separation relies on the use of two different "phases" or "immiscible layers," one of which is held stationary while the other moves over it. Liquid Chromatography is the generic name used to describe any chromatographic procedure in which the mobile phase is a liquid.

The separation occurs because, under an optimum set of conditions, each component in a mixture will interact with the two phases differently relative to the other components in the mixture. HPLC is the term used to describe Liquid Chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector given in the **Fig: 1.4**.

Liquid Chromatography has come a long way with regard to the practical development of HPLC instrumentation and the theoretical understanding of different mechanisms involved in the analyte retention as well as the development of adsorbents with different geometries and surface chemistry.

In HPLC, a liquid sample, or a solid sample dissolved in a suitable solvent, is carried through a Chromatographic column by a liquid mobile phase. Separation is determined by solute / stationary-phase interactions, including liquid–solid adsorption, liquid–liquid partitioning, ion exchange and size exclusion, and by solute / mobile-phase interactions which are listed in **Table: 1.2** with their applications. In each case, however, the basic instrumentation is essentially the same.

Table.1.2: Various Types and Applications of HPLC

TYPE	SAMPLE POLARITY	MOLECULAR WEIGHT RANGE	STATIONARY PHASE	MOBILE PHASE
Adsorption	non-polar to somewhat polar	10^0 - 10^4	silica or alumina	non-polar to polar
Partition (reversed-phase)	non-polar to somewhat polar	10^0 - 10^4	non-polar liquid adsorbed or chemically bonded to the packing material	relatively polar
Partition (normal-phase)	somewhat polar to highly polar	10^0 - 10^4	highly polar liquid adsorbed or chemically bonded to the packing material	relatively non-polar
Ion Exchange	highly polar to ionic	10^0 - 10^4	ion-exchange resins made of insoluble, high-molecular weight solids functionalized typically with sulfonic acid (cationic exchange) or amine (anionic exchange) groups	aqueous buffers with added organic solvents to moderate solvent strength
Size-Exclusion	non-polar to ionic	10^3 – 10^6	small, porous, silica or polymeric particles	polar to non-polar

INSTRUMENTATION:

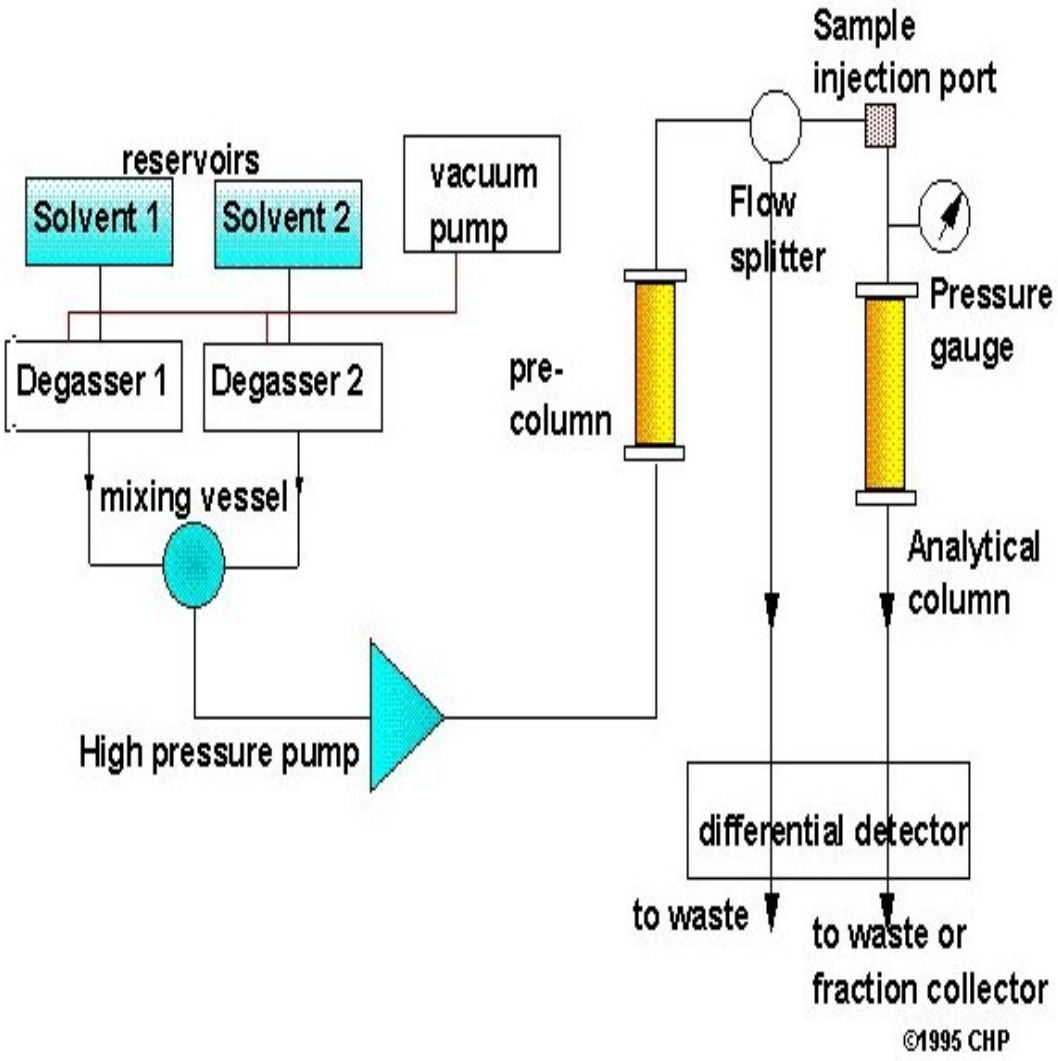


Fig: 1.4. HPLC BASIC INSTRUMENT[15]

Various components involved in HPLC:

1. Solvent delivery systems:

The purpose of the pump, or solvent delivery system, is to ensure the delivery of a precise, reproducible, constant, and pulse-free flow of mobile phase.

There are two classes of HPLC pumps:

1. Constant pressure pumps

2. Constant flow pumps

The most common type of HPLC constant flow pump is the reciprocating piston pump, in which a piston is driven in and out of the solvent chamber by a gear. On the forward stroke, the inlet check valve closes, the outlet check valve opens, and the mobile phase is pumped to the column. On the return stroke, the check valves reverse and solvent is drawn into the chamber. In the single head reciprocating pump, 50 % of the time the mobile phase flows to the column and 50 % of time the chamber is refilling. With the twin-head reciprocating pump the pump heads operate simultaneously but 180^0 out of phase with each other. As a result mobile phase flows to the column 100 % of the time, providing an essentially pulse less flow.

Most separations can be done using isocratic elution which is the use of a single-solvent system that does not change during the analysis. For more complex analysis gradient elution is required. Gradient elution can be generated in three ways. In all cases a computer controlled pumping system is required. In the first phase, controlled amounts of each eluent are metered into a mixing chamber before reaching the high-pressure pump which sends the mixture to the column. In second case the amount of each solvent is regulated by a proportionating valve which is controlled by a microprocessor. The mixed solvent then enters the high pressure pump and flows to the column. In the third case the delivery of high delivery multiple pumps is

controlled individually with a programming device and mixer is send to high-pressure mixing chamber.

2. Columns:

An HPLC typically includes two columns: an analytical column responsible for the separation and a guard column. The guard column is placed before the analytical column, protecting it from contamination.

Guard Columns:

Two problems tend to shorten the lifetime of an analytical column. First, solutes binding irreversibly to the stationary phase degrade the column□s performance by decreasing the available stationary phase. Second, particulate material injected with the sample may clog the analytical column. To minimize these problems, a guard column is placed before the analytical column. Guard columns usually contain the same particulate packing material and stationary phase as the analytical column, but are significantly shorter and less expensive; a length of 7.5 mm and a cost one-tenth of that for the corresponding analytical column are typical. Because they are intended to be sacrificial, guard columns are replaced regularly.

Analytical Columns:

The most commonly used columns for HPLC are constructed from stainless steel with internal diameters between 2.1 mm and 4.6 mm, and lengths ranging from approximately 30 mm to 300 mm. These columns are packed with 3–10 mm porous silica particles that may have an irregular or spherical shape. Typical column efficiencies are 40,000–60,000 theoretical plates/m. Micro columns use less solvent and, because the sample is diluted to a lesser extent, produce larger signals at the detector. These columns are made from fused silica capillaries with internal diameters of 44–200 mm and lengths of up to several meters.

Micro columns packed with 3–5 mm particles have been prepared with column efficiencies of up to 250,000 theoretical plates. Open tubular micro columns also have been developed, with internal diameters of 1–50 mm and lengths of approximately 1 m. These columns, which contain no packing material, may be capable of obtaining column efficiencies of up to 1 million theoretical plates.

The development of open tubular columns, however, has been limited by the difficulty of preparing columns with internal diameters less than 10 mm.

3. Stationary Phases:

In Liquid–Liquid Chromatography the stationary phase is a liquid film coated on a packing material consisting of 3–10 mm porous silica particles. The stationary phase may be partially soluble in the mobile phase, causing it to "bleed" from the column over time. To prevent this loss of stationary phase, it is covalently bound to the silica particles. Bonded stationary phases are attached by reacting the silica particles with an organochlorosilane of the general form Si $(CH_3)_2RCl$, where R is an alkyl or substituted alkyl group.

To prevent unwanted interactions between the solutes and any unreacted –SiOH groups, the silica frequently is "capped" by reacting it with Si $(CH_3)_3Cl$; such columns are designated as end-capped. The properties of a stationary phase are determined by the nature of the organosilane□s alkyl group. If R is a polar functional group, then the stationary phase will be polar. Since the stationary phase is polar, the mobile phase is a nonpolar or moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called normal phase Chromatography.

In reverse phase Chromatography, which is the more commonly encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane for which the R group

is an n-octyl (C_8) or n-octyldecyl (C_{18}) hydrocarbon chain. Most reverse phase separations are carried out using a buffered aqueous solution as a polar mobile phase.

4. Mobile Phases:

The elution order of solutes in HPLC is governed by polarity. In a normal-phase separation the least polar solute spends proportionally less time in the polar stationary phase and is the first solute to elute from the column.

The mobile phases used in normal-phase chromatography are based on nonpolar hydrocarbons, such as hexane, heptane, or octane, to which is added a small amount of a more polar solvent, such as 2-propanol. Solvent selectivity is controlled by the nature of the added solvent. Additives with large dipole moments, such as methylene chloride and 1,2-dichloroethane, interact preferentially with solutes that have large dipole moments, such as nitro-compounds, nitriles, amines, and sulfoxides. Good proton donors such as Chloroform, m-Cresol, and Water interact preferentially with basic solutes such as amines and Sulfoxides, whereas good proton acceptors such as alcohols, ethers, and amines tend to interact best with hydroxylated molecules such as acids and phenols. A variety of solvents used as mobile phases in normal-phase Chromatography are listed in **Table: 1.3**, some of which may need to be stabilized by addition of an antioxidant, such as 3-5 % Ethanol, because of the propensity for peroxide formation.

In a reverse-phase separation the order of elution is reversed, with the most polar solute being the first to elute. The mobile phases used in reversed-phase Chromatography are based on a polar solvent, typically water, to which a less polar solvent such as Acetonitrile or Methanol is added. Solvents with large dipole moments, such as methylene chloride and 1,2-dichloroethane, interact preferentially with solutes that have large dipole moments, such as nitro- compounds, nitriles,

amines, and sulfoxides. Solvents that are good proton donors, such as Chloroform, m-Cresol, and Water, interact preferentially with basic solutes such as amines and Sulfoxides, and solvents that are good proton acceptors, such as alcohols, ethers, and amines, tend to interact best with hydroxylated molecules such as acids and phenols.

Table: 1.3. List of solvents used in HPLC

Solvent	Adsorption energy(e^0) on Al_2O_3	Solvent	Adsorption energy(e^0) on Al_2O_3
n-Pentane	0.00	Acetone	0.56
Isooctane	0.01	Ethyl Acetate	0.58
Cyclohexane	0.04	Dimethylamine	0.63
Carbon Tetrachloride	0.18	Acetonitrile	0.65
Toluene	0.29	Ethanol	0.88
Benzene	0.32	Methanol	0.95
Chloroform	0.40	Acetic Acid	Large
Methyl Ethyl Ketone	0.51	Water	Very large

5. Detectors:

The detection of UV light absorbance offers both convenience and sensitivity for molecules. When a chromophore, the wavelength of detection for a drug should be based on its UV spectrum in the mobile phase and not in pure solvents, the most selective wavelength for detecting a drug is frequently the longest wavelength maximum to avoid interference from solvents, buffers and excipients. Other method of detection can be useful are required in some instances.

- Solute specific detectors (UV, Visible, Fluorescence, Electrochemical, IR, Radioactivity).
- Bulk property detectors (Refractive index, Viscometric, Conductivity).

- Desolvation detectors (Flame ionization etc.).
- LC-MS detectors.
- Reaction detectors.

SYSTEM SUITABILITY PARAMETERS[16]:

System suitability test are an integral part of Gas and Liquid Chromatography. They are used to verify that the resolution and reproducibility of the Chromatographic system are adequate for the analysis to be done. These tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.

There are numerous guidelines which detail the expected limits for typical Chromatographic methods. In the current FDA guideline on "Validation of Chromatographic methods" the following acceptance limits are proposed as initial criteria.

1. Capacity factor (k'):

$k' = (t_R - t_0) / t_0$

The capacity factor is a measure of the degree of retention of an analyte relative to an unretained peak.

Where, t_R - retention time for the sample peak.

t_o - retention time for the unretained peak.

The peak should be well-resolved from other peaks and the void volume. Generally the value of k' is > 2

2. Backpressure:

The pressure required to pump the mobile phase through the column. It is related to mobile phase viscosity (η), flow rate (F), column length (L), and diameter (d_c), and

particle size (d_p) by the following equation:

$$\Delta P \alpha FL\eta / d_p^2 d_c^2$$

3. Resolution (R_s):

Ability of a column to separate chromatographic peaks, Resolution can be improved by increasing column length, decreasing particle size, increasing temperature, changing the eluent or stationary phase. It can also be expressed in terms of the separation of the apex of two peaks divided by the tangential width average of the peaks.

$$R_s = \Delta t_R / 0.5 (W_1 + W_2);$$

Where $\Delta t_R = t_2 - t_1$For reliable quantitation, well-separated peaks are essential for quantitation. R_s of > 2 between the peak of interest and the closest potential interfering peak (impurity, excipients, degradation product, internal standard, etc.) are desirable.

4. Theoretical plate number / Efficiency (N):

A measure of peak band spreading determined by various methods, some of which are sensitive to peak asymmetry. The most common are shown here, with the ones most sensitive to peak shape shown first.

4-sigma / tangential

$N = 16 (t_R / W)^2 = L / H$

Half height

$N = 5.54 (t_R / W)^2 = L / H$

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram.

Where, t_R - Retention time for the sample peak.

W - Peak width.

N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H (height), or HETP (height equivalent of a theoretical plate), measures the column efficiency per unit length (L) of the column. Parameters which can affect N or H include.

Peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte.

The theoretical plate number depends on elution time but in general should be > 2000.

5. Tailing factor (T):

A measure of the symmetry of a peak.

$T = W_{0.05} / 2f$

Where, $W_{0.05}$ - Peak width at 5 % height

f - Distance from peak front to apex point at 5 % height.

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak.

Limits -**T** < / = 2

PART C: VALIDATION[17-22]:

Validation may be viewed as the establishment of an experimental data base that certifies an analytical method performs in the manner for which it was intended and is the responsibility of the method development laboratory. Method transfer, on the other hand, is the introduction of a validated method into a designated so that it can be used in the same capacity for which it was originally developed.

Validation is a basic requirement to ensure quality and reliability of the results for all analytical applications.

Validation is defined as follows by different agencies:

European Committee (EC):

Action of providing in accordance with the principles of good manufacturing practice that any procedure, process, equipment, material, activity or system actually leads to the expected results. In brief validation is a key process for effective Quality Assurance.

Food and Drug Administration (FDA):

Provides a high degree of assurance that specific process will consistently produce a product meeting its predetermined specification and quality attributes.

World Health Organization (WHO):

Action of providing that any procedure, process, equipment, material, activity or system actually leads to the expected results.

Analytical method validation:

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated.

Before their introduction into routine use

- Whenever the conditions change for which the method has been validated, e.g., instruments with different characteristics.
- Whenever the method is changed, and the change is outside the original scope of the method. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics.

  The parameters as defined by the ICH and by other organizations are;

Precision:

"The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels; repeatability, intermediate precision and reproducibility."

Precision should be obtained preferably using authentic samples. As parameters, the standard deviation (SD), the relative standard deviation (coefficient of variation) and the confidence interval should be calculated for each level of precision.

Repeatability expresses the analytical variability under the same operating conditions over a short interval of time (within-assay, intra-assay). At least nine determinations covering the specified range or six determinations at 100 % test concentration should be performed. Intermediate precision includes the influence of additional random effects within laboratories, according to the intended use of the procedure, for example, different days, analysts or equipment, etc.

Reproducibility, i.e., the precision between laboratories (collaborative or interlaboratory Studies), is not required for submission, but can be taken into account for standardization of analytical procedures.

Specificity:

"Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual procedure may be compensated by other supporting analytical procedure(s)".

With respect to identification, discrimination between closely related compounds likely to be present should be demonstrated by positive and negative samples. In the case of chromatographic assay and impurity tests, available impurities / degradants

can be spiked at appropriate levels to the corresponding matrix or else degraded samples can be used. For assay, it can be demonstrated that the result is unaffected by the spiked material. Impurities should be separated individually and/or from other matrix components. Specificity can also be demonstrated by verification of the result with an independent In the case of chromatographic separation, resolution factors should be obtained for critical separation. Tests for peak homogeneity, for example, by diode array detection (DAD) or mass spectrometry (MS) are recommended.

Accuracy:

"The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found".

Accuracy can be demonstrated by the following approaches:

- Inferred from precision, linearity and specificity
- Comparison of the results with those of a well characterized, independent procedure
- Application to a reference material (for drug substance)
- Recovery of drug substance spiked to placebo or drug product (for drug product)
- Recovery of the impurity spiked to drug substance or drug product (for impurities)

For the quantitative approaches, at least nine determinations across the specified range should be obtained, for example, three replicates at three concentration levels each. The percentage recovery or the difference between the mean and the accepted true value together with the confidence intervals are recommended.

It is important to use the same quantitation method (calibration model) in the accuracy studies as used in the control test procedure. Sometimes in the literature, the data from

linearity studies are simply used to calculate the content of spiked samples. However, the validation linearity study is usually not identical to the calibration applied in routine analysis. Again, validation has to demonstrate the suitability of the routine analytical procedure. Deviations from the theoretical recovery values, while performing a calibration with a drug substance alone, may indicate interferences between the analyte and placebo components, incomplete extraction, etc. In such a case, the calibration should be done with a synthetic mixture of placebo and drug substance standard. Such interferences will also be detected by comparing the linearity□s of diluted drug substance and of spiked placebo, but the evaluation is more complex. In contrast, recovery studies usually concentrate directly on the working range and are simpler (but not always easy) to evaluate.

Linearity:

"The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample".

It may be demonstrated directly on the analyte, or on spiked samples using at least five concentrations over the whole working range. Besides a visual evaluation of the analyte signal as a function of the concentration, appropriate statistical calculations are recommended, such as a linear regression. The parameters slope and intercept, residual sum of squares and the coefficient of correlation should reported. A graphical presentation of the data and the residuals is recommended.

Range:

"The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of

precision, accuracy and linearity."

Limit of detection (LOD):

"The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest concentration of analyte in a sample which can be quantitatively determined with suitable precision and accuracy."

Various approaches can be applied:

- Visual definition
- Calculation from the signal-to-noise ratio (LOD and LOQ correspond to 3 or 2 and 10 times the noise level, respectively)
- Calculation from the standard deviation of the blank

Calculation from the calibration line at low concentrations

LOD; LOQ ¼ F_SD b (2.6-1)

F: factor of 3.3 and 10 for LOD and LOQ, respectively

SD: standard deviation of the blank, standard deviation of the ordinate intercept, or residual standard deviation of the linear regression

b: slope of the regression line

The estimated limits should be verified by analyzing a suitable number of samples containing the analyte at the corresponding concentrations. The LOD or LOQ and the procedure used for determination, as well as relevant chromatograms, should be reported.

Limit of Quantitation (LOQ):

The quantitation limit is the lowest level of analyte that can be accurately and precisely measured. This limit is required only for impurity methods and is

determined by reducing the analyte concentration until a level is reached where the precision of the method is unacceptable. If not determined experimentally, the quantitation limit is often calculated as the analyte concentration that gives S / N = 10. An example of quantitation limit criteria is that the limit will be defined as the lowest concentration level for which an RSD 20 % is obtained when an intra-assay precision study is performed.

Robustness:

According to ICH Q2A [1a] "the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage".

Furthermore, it is stated in ICH Q2B [1b], "The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used".

Ruggedness:

"The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different days, etc. Ruggedness is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method. Ruggedness is a

measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst". The degree of reproducibility is then evaluated by comparison of the results obtained under varied conditions with those under standard conditions.

RUFINAMIDE DEFINITION:

Rufinamide is an anticonvulsant medication. It is used in combination with other medication and therapy to treat Lennox–Gastaut syndrome and various other seizure disorders. Rufinamide is a triazole derivative.

RUFINAMIDE INTRODUCTION:

Rufinamide was approved by the US Food and Drug Administration on November 14, 2008 as adjunctive treatment of seizures associated with Lennox-Gastaut syndrome in children 4 years and older and adults. Its official FDA-approved labeling does not mention use in the treatment of partial seizures in as much as clinical trials submitted to the FDA were marginal. However, several recent clinical trials suggest that the drug has efficacy for partial seizures

MECHANISM OF ACTION OF RUFINAMIDE:

The mechanism of action of rufinamide is unknown. There is some evidence that rufinamide can modulate the gating of voltage-gated sodium channels, a common target for antiepileptic drugs. A recent study indicates subtle effects on the voltage-dependence of gating and the time course of inactivation in some sodium channel isoforms that could reduce neuronal excitability. However, this action cannot explain the unique spectrum of activity of rufinamide.

DRUG PROFILE[23, 24]:

Name : Rufinamide.

Chemical Name : 1-(2, 6-difluorobenzyl)-1H-1,2,3-triazole-4-carboxamide..

Molecular Formula : $C_{10}H_8F_2N_4O$

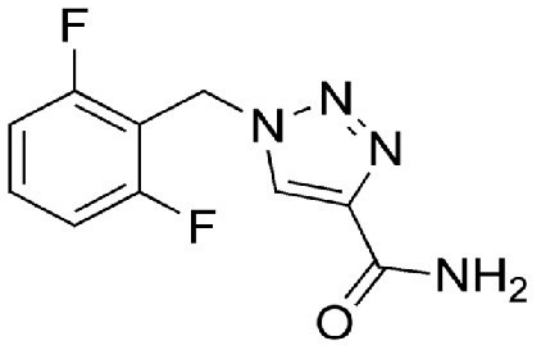
Molecular Weight : 238.19g / mol.

PHYSIOCHEMICAL PROPERTIES:

Nature : White to off-white crystalline powder.

Solubility : Practically insoluble in water, slight solubility in tetra hydro furan (THF) and methanol, very slight solubility in ethanol and acetonitrile, soluble in dimethyl sulfoxide (DMSO).

STRUCTURE:



Category : Anticonvulsant drug.

Storage condition : Store in well closed, light resistant containers in a cool place.

Functional groups : Amide group, secondary and tertiary amino groups.

2. OBJECTIVES

A very few analytical methods appeared in the literature for the determination of Rufinamide are generally based HPLC, Temperature - programmed packed Capillary Liquid Chromatography, HPTLC methods. HPLC - Electrospray Ionization Mass Spectrometry (HPLC-MS / ESI).

In the present work, an attempt was made to provide newer, simple, accurate and low cost Spectrophotometry methods namely Zero order Spectrophotometry method, including Area under curve method and one HPLC method for the effective quantitative determination of Rufinamide as an active pharmaceutical ingredient as well as in pharmaceutical preparations without the interferences of other constituent in the formulations.

Validation of the method was done in accordance with USP and ICH guideline for the assay of active ingredients. The methods were validated for parameters like accuracy, linearity, precision, specificity, ruggedness, robustness, and system suitability. These methods provide means to separate the components characterize and quantify the components. These proposed methods are suitable for the pharmaceutical analysis in analytical laboratories.

In summary, the primary objective of proposed work was to:

- Develop new, simple, sensitive, accurate, and economical analytical methods for the estimation of Rufinamide.
- Validate the proposed methods in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of ufinamide in its dosage form.

3. REVIEW OF LITERATURE

1. Development and validation of an HPLC-UV detection assay for the determination of rufinamide in human plasma and saliva[25].

***Mazzucchelli I** et al.*

The development of a simple and rapid high-performance liquid chromatography (HPLC) method for the determination of the new antiepileptic drug rufinamide (RFN) in human plasma and saliva is reported. Samples (250 μl) are alkalinized with ammonium hydroxide (pH 9.25) and extracted with dichloromethane using metoclopramide as internal standard. Separation is achieved with a Spherisorb silica column (250 × 4.6 mm i.d., 5 μm) at 30 °C using as mobile phase a solution of methanol/dichloromethane/n-hexane 10/25/65 (vol/vol/vol) mixed with 6 ml ammonium hydroxide. The instrument used was a Shimadzu LC-10Av chromatograph and flow rate was 1.5 ml min(-1), with a LaChrom L-7400 UV detector set at 230 nm. Calibration curves are linear [r(2) = 0.998 ± 0.002 for plasma (n = 10) and r(2) = 0.999 ± 0.001 for saliva (n = 9)] over the range of 0.25-20.0 μg ml(-1), with a limit of quantification at 0.25 μg ml(-1). Precision and accuracy are within current acceptability standards. The assay is suitable for pharmacokinetic studies in humans and for therapeutic drug monitoring.

2. Rapid assay of rufinamide in dried blood spots by a new liquid chromatography-tandem mass spectrometric method[26].

Giancarlo la Marca *et al.*

Rufinamide (RUF) is a new antiepileptic drug with efficacy in several types of seizures. The aim of this study was to evaluate the use of dried blood spot (DBS)

specimens to determinate RUF levels during treatment. Therapeutic drug monitoring of RUF could be useful in routine clinical practice. Advantages of DBS include short collection time, low invasiveness, ease and low cost of sample collection, transport and storage. The analysis was performed in selected reaction monitoring (SRM) mode. The calibration curve in matrix was linear in the concentration range of 0.008-0.8 mg/L (0.48-47.60 mg/L in DBS) of rufinamide with correlation coefficient value of 0.996. In the concentration range of 0.48-47.6 mg/L, the coefficients of variation in DBS were in the range 1.58-4.67% and the accuracy ranged from 89.73% to 107.32%. The sensitivity and specificity of tandem mass spectrometry allow now high throughput rufinamide analysis. This new assay has favourable characteristics being highly precise and accurate. The published HPLC-UV methods also proved to be precise and accurate, but required not less than 0.2-0.5 mL of plasma and are therefore unsuitable for sample collection in neonates in whom obtaining larger blood samples is not convenient or possible.

3. Simultaneous HPLC-UV analysis of rufinamide, zonisamide, lamotrigine, oxcarbazepine monohydroxy derivative and felbamate in deproteinized plasma of patients with epilepsy[27].

Contin M *et al*,

We present an implementation of a method we previously reported allowing the newer antiepileptic drugs (AEDs) rufinamide (RFN) and zonisamide (ZNS) to be simultaneously determined with lamotrigine (LTG), oxcarbazepine's (OXC) main active metabolite monohydroxycarbamazepine (MHD) and felbamate (FBM) in plasma of patients with epilepsy using high performance liquid chromatography (HPLC) with UV detection. Plasma samples (250 microL) were deproteinized by 1

mL acetonitrile spiked with citalopram as internal standard (I.S.). HPLC analysis was carried out on a Synergi 4 microm Hydro-RP, 250 mm x 4.6 mm I.D. column. The mobile phase was a mixture of potassium dihydrogen phosphate buffer (50 mM, pH 4.5), acetonitrile and methanol (65:26.2:8.8, v/v/v) at an isocratic flow rate of 0.8 mL/min. The UV detector was set at 210 nm. The chromatographic run lasted 19 min. Commonly coprescribed AEDs did not interfere with the assay. Calibration curves were linear for both AEDs over a range of 2-40 microg/mL for RFN and 2-80 microg/mL for ZNS. The limit of quantitation was 2 microg/mL for both analytes and the absolute recovery ranged from 97% to 103% for RFN, ZNS and the I.S. Intra- and interassay precision and accuracy were lower than 10% at all tested concentrations. The present study describes the first simple and validated method for RFN determination in plasma of patients with epilepsy. By grouping different new AEDs in the same assay the method can be advantageous for therapeutic drug monitoring (TDM).

4. Practice of solid-phase extraction and protein precipitation in the 96-well format combined with high-performance liquid chromatography–ultraviolet detection for the analysis of drugs in plasma and brain[28]

M. C. Rouan *et al*,

C18 Empore 96-well extraction disc plates have been employed for the analysis of three drugs with different polarities in plasma in conjunction with HPLC–UV, rufinamide, ICL670 and an anticonvulsant agent (AA1) in an early stage of development. With the most polar compound (AA1), ion-pair extraction at pH 12 was applied. The method developed for the assay of AA1 in plasma was applied to its determination in brain using an Oasis HLB plate following homogenisation in a pH

7.4 buffer and protein precipitation with NaOH–ZnSO4, thereby saving time for method development. Protein precipitation in the 96-well format with filtration of the precipitate was applied to the determination of ICL670, a highly protein-bound compound (>99.5%), with a good recovery (78%). Reversed-phase chromatography was applied using a short 5 cm column packed with 3 μm particles for the determination of ICL670 and AA1 and two parallel columns (15 cm long) for the determination of rufinamide. The methods were used routinely, one plate per analysis day being processed, resulting in increase in sample throughput and saving in solvents.

4. METHODOLOGY

PART A: UV SPECTROSCOPY

METHOD A: ZERO ORDER DERIVATIVE SPECTROSCOPY

Selection of analytical wavelength:

Appropriate dilutions were prepared for drug from the standard stock solution and the solutions were scanned in the wavelength range of 200-400 nm. The absorption spectra thus obtained were derivatized from Zero order and AUC method.

Preparation of stock solutions:

Standard Rufinamide 100 mg was weighed and transferred to a 10 ml volumetric flask and dissolved in dmso. The flask was shaken and volume was made up to the mark with dmso to give a solution containing 10000 mcg / ml.

Selection of analytical concentration ranges:

From the standard stock solution of Rufinamide, appropriate aliquots were pipetted out in to 10 ml volumetric flasks and dilutions were made with distilled water to obtain working standard solutions of concentrations from 100 to 600 mcg / ml. Absorbance for these solutions were measured at 262 nm. For the standard solution analytical concentration range were found to be 100-600 mcg / ml and those values were reported in **Table: 5.1.**

Calibration curve for the Rufinamide (100 - 600 mcg / ml):

Appropriate volume of aliquots from standard Rufinamide stock solutions were transferred to different volumetric flasks of 10 ml capacity. The volume was adjusted to the mark with distilled water to obtain concentrations of 100, 200, 300, 400, 500 and 600 mcg / ml. Absorbance spectra of each solution against distilled water as blank were

measured at 262 nm and the graphs of absorbance against concentration was plotted and are shown in **Fig: 5.1.** The regression equation and correlation coefficient was determined and are presented in **Table: 5.2.**

Sample preparation for determination of Rufinamide from dosage form:

Twenty tablets (Banzel) were weighed and finely powdered. The powder equivalent to 25 mg of Rufinamide was weighed accurately and transferred into a 25 ml standard volumetric flask. The contents were dissolved in dmso and sonicated for five minutes. This solution was made upto the mark with dmso to get the solution of 1000 mcg / ml. An aliquot of 1 ml of test solution was diluted to 10 ml with Water in 10 ml standard volumetric flask to produce the concentration 100 mcg / ml. carefully filtered through 0.45 micron Whatmann filter paper (No. 41) and used for estimation of Rufinamide.

Validation of spectrophotometric method

Linearity and Range:

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample with in a given range and was given in **Fig: 5.2.** The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

Precision:

The precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogenous samples. It provides an indication of random error results and was expressed as %RSD.

Accuracy:

Accuracy is the closeness of the test results obtained by the method to the true value. To study the accuracy, 20 tablets were weighed and powdered and analysis of the same was carried out. Recovery studies were carried out by adding known amount of standard drug solution (400 mcg / ml) to the sample solution. The % recovery was calculated and reported in **Table: 5.3.**

Intra and inter-day precision:

A Variation of results within the same day (intra-day), variation of results between days (inter-day) was analyzed. Intra-day precision was determined by analyzing Rufinamide for six times in the same day at 262 nm. Inter-day precision was determined by analyzing daily once for six days at 262 nm and % RSD was calculated and were shown in **Table: 5.4.**

Ruggedness:

The solutions were prepared and analyzed with change in the analytical conditions like different laboratory conditions and different analyst and reported in **Table: 5.5.**

METHOD B: AREA UNDER CURVE METHOD

Preparation of standard stock solutions:

Preparation of standard stock solution was same as described in method A.

Selection of analytical wavelength range for Area Under Curve:

Appropriate dilutions were prepared for drug from the standard stock solution and the solutions were scanned in the wavelength range of 200 - 400 nm. The absorption spectra obtained was showing the absorption maxima [λ_{max}] at 262 nm and Area Under Curve [AUC] in absorption spectra were measured between the wavelength range 254 to 270 nm which illustrated in **Fig: 5.3**.

Selection of analytical concentration range:

Selection of analytical concentration range was made same as described in method A by measuring AUC between 254 nm to 270 nm instead of absorbance at 262 nm. For the standard solutions analytical concentration range was found to be 100 - 600 µg / ml and those values were reported in **Table: 5.6**.

Calibration curve for the Rufinamide (100 - 600 µg / ml):

Calibration curve for the Rufinamide was prepared same way as described in method A by measuring AUC between 254 nm to 270 nm instead of absorbance at 262 nm and is shown in **Fig: 5.4**. The regression equation and correlation coefficient were determined which are presented in **Table: 5.7**.

Sample preparation for determination of Rufinamide from dosage form:

Sample preparation for determination of Rufinamide from dosage form was same as described in method A.

Validation of Spectrophotometric method All the validation parameters are same as described in Method A.

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In the present investigation, we have developed a simple and sensitive RP-HPLC method for quantitative estimation of Rufinamide in bulk drug and pharmaceutical formulations.

Experimental:

Instrumentation:

An isocratic high performance liquid chromatography equipped with Auto Sampler and DAD or PDA detector, with software EZ Chrome Elite was used. Qualisil gold C18 Column was used (4.6 x 150mm, 5 µm).

Reagents:

The reference standard Rufinamide was kindly gifted by Ranbaxy Laboratories (Ahmadabad, India). The standard drugs were used without further purification. Methanol and other reagents of HPLC grade was procured from Qualigens, Mumbai were used throughout the experiment. The mobile phase consists of mixture of Methanol and Water in the ratio of 50 : 50 (v / v).

Preparation of working stock solution of Rufinamide:

Accurately weigh and transfer 10mg of Rufinamide Working standard into a 10 mL volumetric flask add about 5 mL of Diluent and sonicate to dissolve it completely a make volume up to the mark with the same solvent (Stock solution). Further pipette 0.4 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Chromatographic conditions:

Equipment	**:** Agilent High performance liquid chromatography equipped with Auto Sampler and DAD or PDA detector.
Column	**:** Qualisil gold C18 (4.6 x 150mm, 5 µm)
Flow rate	**:** 1 ml/ min
Wavelength	**:** 220 nm
Injection volume	**:** 20 µl
Column oven	**:** Ambient
Run time	**:** 8.0 min

Assay procedure:

Working standard solutions containing 10 to 60 mcg / ml of Rufinamide were prepared by appropriate dilution of the stock solution with the mobile phase. Twenty µl aliquot of each solution was injected into the column for five times and the chromatograms were

recorded and are presented in **Fig: 5.5**. The retention time was found to be 5.20 min. Calibration graph was constructed by plotting the mean peak area as a function of Rufinamide concentration.

Analysis of formulation:

Twenty tablets (Brand X) were accurately weighed and finely powdered. Tablet powder equivalent to 0.01 mg of Rufinamide was weighed accurately and dissolved in 50 ml of Methanol in a 100 ml volumetric flask and diluted up to the mark with Water to get the concentration of 1000 mcg / ml. From this, pipetted out 0.4 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with the mobile phase to get concentration 40 mcg / ml was prepared for RP-HPLC method. Resultant solution was filtered through Whatman filter paper. The final solution was injected into chromatographic system for three times.

VALIDATION OF ANALYTICAL METHOD:

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.

1. Accuracy:

The accuracy of a method was inferred by establishing the precision and linearity of the standards and given in **Table: 5.12.**

2. Precision:

The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intra-day studies, six repeated injections of standard solution was made and the response factor of drug peak and % RSD were calculated and present in

Table: 5.13. The chromatogram was shown in **Fig: 5.6**. In the inter-day variation studies, six repeated injections of standard solution were made for six consecutive days and response factor of drugs peak and % RSD were calculated shown in **Table: 5.13.** From the data obtained, the developed method was found to be precise.

3. Linearity:

The linearity of the method was demonstrated over the concentration range of 10- 60 mcg / ml of the target concentration. Aliquots of 10, 20, 30, 40, 50 and 60 mcg / ml were prepared from above prepared stock solution. Different concentrations of the pure drug were injected into the chromatographic system. Calibration curve of Rufinamide was constructed by plotting peak area vs. applied concentration of Rufinamide. The obtained results shown an excellent correlation between peak area and concentration of pure drug within the concentration range and it has shown in **Fig: 5.7.** The correlation coefficient for the average area at each level versus concentration of analyte was calculated and is presented in **Table: 5.14.** and their calibration parameters were shown in **Table: 5.15.**

$$\text{Standard deviation (SD)} = \quad \sigma = \sqrt{\frac{\Sigma(x - x_1)^2}{n-1}}$$

Where, x = sample

x_1 = mean value of samples ; n = number of samples.

The correlation Coefficient and Percentage curve fittings were calculated by using the following formula:

$$R = \sum (X-X)\,(Y-Y) / (n-1)\,Sx\,Sy$$

Where, X = Concentration

Y = Instrumental response

Sx = Standard deviation of x

Sy = Standard deviation of y

Percentage Curve Fitting = 100 X Correlation Coefficient.

4. Limit of detection (LOD):

Limit of detection is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. From the standard stock solution 0.4 ml was pipetted out into 10 ml volumetric flask and the volume was made up to the mark with mobile phase. From this solution, Pipetted out 1ml of 100 mcg / ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Final dilution was made by pipetting 1.51 ml (10 mcg / ml) of above solution into a 10 ml of volumetric flask and diluted up to the mark with diluent. The solution was injected and chromatogram was shown in **Fig: 5.8.** The Limit of detection was found to be 1.51 mcg / ml for Rufinamide. The results of LOD were shown in **Table: 5.17.**

5. Limit of quantitation (LOQ):

Based on the LOD strength (1.51 mcg / ml, standard solution), the LOQ values were calculated by multiplication with three times.

From the standard stock solution 0.4 ml was pipetted out was placed into 10 ml volumetric flask and volume was made up to the mark with mobile phase. Pipetted out 1ml of 100 mcg / ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent. Further pipetted 4.60 ml of above diluted solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. The solution was injected and chromatogram was shown in **Fig: 5.9.** The Limit of quantitation was found to be 4.60 mcg / ml for Rufinamide. The results of LOQ were shown in **Table: 5.18.**

6. Ruggedness:

The ruggedness of an analytical method is degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures and different days, etc. Rufinamide sample equivalent to 0.01 mg was weighed and dissolved in a 100 ml volumetric flask containing mobile phase(50 ml), sonicated for few mints and the final volume was made with mobile phase. The samples were injected into the column; chromatogram was recorded and was shown in **Fig: 5.10.** The results of ruggedness were shown in the **Table: 5.19.**

7. Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of robustness, a number of method parameters such as pH, flow rate, column temperature, injection volume, detection wavelength, or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. The sample was analyzed separately by slightly changes in the analytical method as given below:

By changing the flow rate of mobile phase to 1.0 ± 0.1 ml / min. The chromatograms were recorded and were shown in **Fig: 5.11. and 5.12.** The retention time values were measured. The robustness results were shown in **Table: 5.21. and 5.22**.

By changing ratio of the mobile phase i.e. Water : Methanol from 50 : 50 to 48: 52 and 52 : 48. The chromatograms were recorded and were shown in **Fig: 5.13. and 5.14**.

The retention time values were measured. The robustness results were shown in **Table: 5.24**. **and 5.25.**

By changing ratio of the wavelength i.e. Water : Methanol from 220 ± 3nm. The chromatograms were recorded and were shown in **Fig: 5.15. and 5.16**. The retention time values were measured. The robustness results were shown in **Table: 5.27**. **and 5.28.**

8. System suitability

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (R_t), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 100 mcg / ml. The results given in **Table: 5.16.** were within acceptable limits.

5. RESULTS

METHOD A: ZERO ORDER DERIVATIVE SPECTROSCOPY

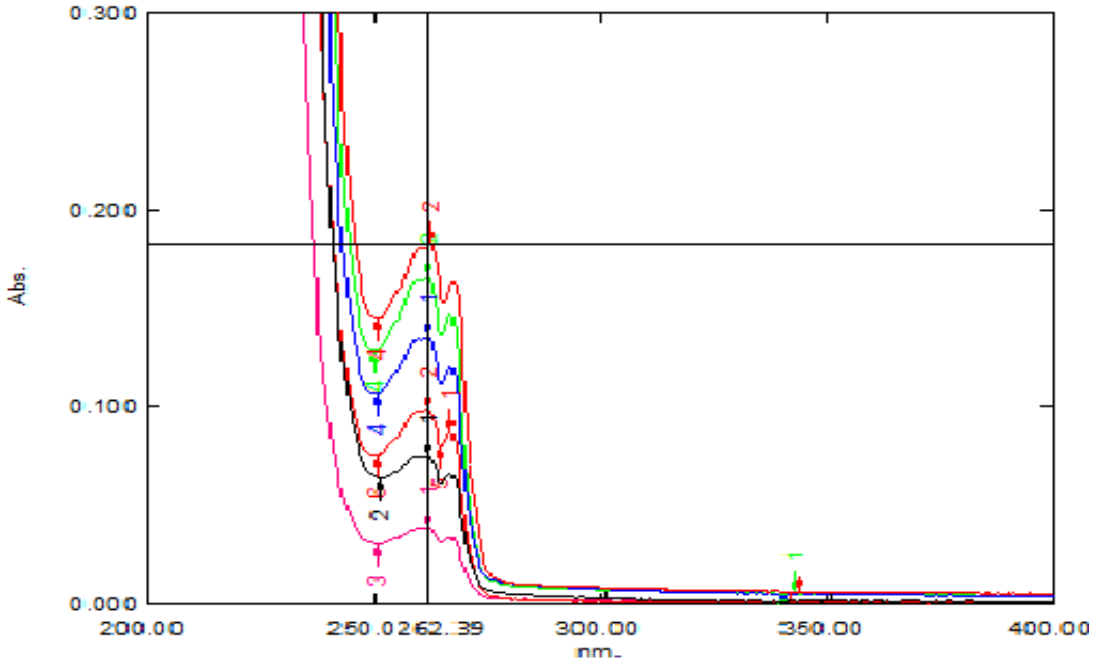


Fig : 5.1 Zero order spectra of Rufinamide showing absorbance at 262 nm

Table : 5.1

Results of calibration curve at 262 nm for Rufinamide by Zero order Spectroscopy

S. No.	Concentration (mcg / ml)	Absorbance at 262 nm
1	100	0.030
2	200	0.062
3	300	0.091
4	400	0.122
5	500	0.152
6	600	0.181

Table : 5.2

Optimum conditions, Optical characteristics and Statistical data of the Regression equation in Zero order Spectroscopy

Parameter	UV Method
λ_{max} (nm)	262
Beer's law limits (mcg/ml)	100-600
Molar extinction coefficient (L mol^{-1} cm^{-1})	0.0003×10^4
Sandell's sensitivity (mcg/cm^2-0.001 absorbance units)	3.305
Regression equation (Y*)	Y = 0.0003 C + 0.00075
Slope (b)	0.0003
Intercept (a)	0.00075
Correlation coefficient(r^2)	0.999
Intraday Precision (% RSD**) Interday Precision (% RSD**)	0.678 0.748
Limit of detection (mcg/ml)	3.44
Limit of quantitation (mcg/ml)	10.45

*Y = bx + a where x is the concentration of Rufinamide in mcg / ml and Y is the absorbance at the respective λmax.

**Average of Six determinations

Table : 5.3

Determination of Accuracy results for Rufinamide at 262 nm by Zero order Spectroscopy

Brand used	Amount of sample (mcg / ml)	Amount of drug added (mcg / ml)	Amount Recovered	% Recovery ± SD**	% RSD
BANZEL	400	200	201.82	100.89 ±0.46	0.45
	400	400	396.34	99.09 ± 0.48	0.48
	400	600	601.82	100.32 ± 17	0.17

**Average of Six determinations

Table: 5.4

Determination of Precision for Rufinamide at 262 nm by Zero order derivative spectroscopy

Conc. mcg / ml	Intra-day Absorbance Mean ± SD**	% RSD	Inter-day Absorbance Mean ± SD**	% RSD
100	0.0295 ± 0.00055	1.85	0.029 ± 0.00055	1.85
200	0.06185 ± 0.00075	1.21	0.061 ± 0.0089	1.46
300	0.0901 ± 0.00075	0.83	0.09 ± 0.0089	0.99
400	0.1203 ± 0.00082	0.67	0.12 ± 0.00089	0.74
500	0.152 ± 0.00126	0.83	0.152 ± 0.0011	0.76

**Average of six determinations

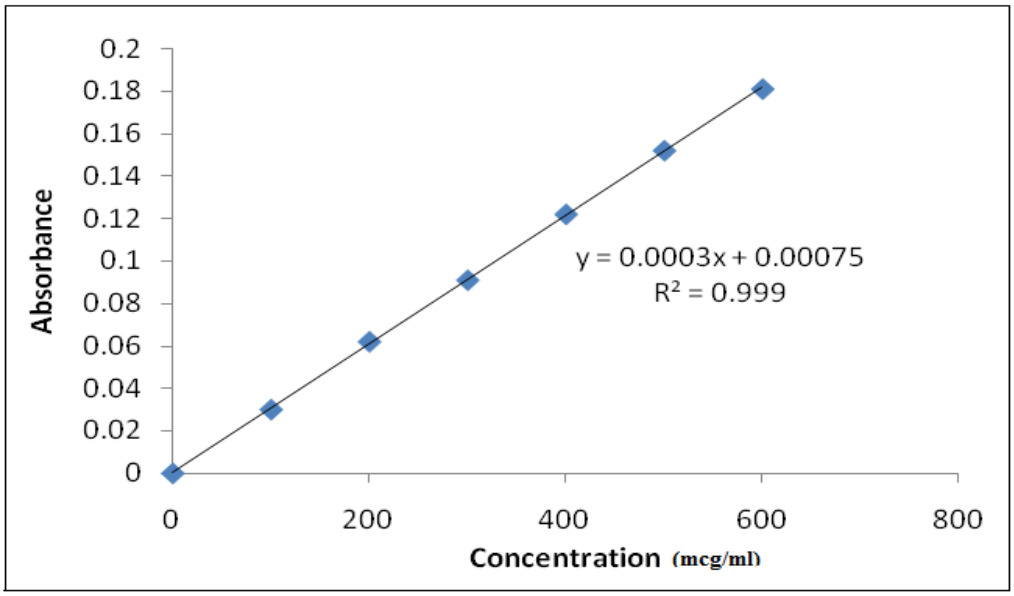


Fig. 5.2. Linearity curve for Rufinamide at 262 nm by Zero order Spectroscopy

Table: 5.5.

Ruggedness results for Rufinamide at 262 nm by Zero order derivative spectroscopy

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
BRAND X	400	398.087	98.087±0.48	399.52	99.52 ± 0.34

**Average of Six determinations

METHOD B: AREA UNDER CURVE SPECTROSCOPY

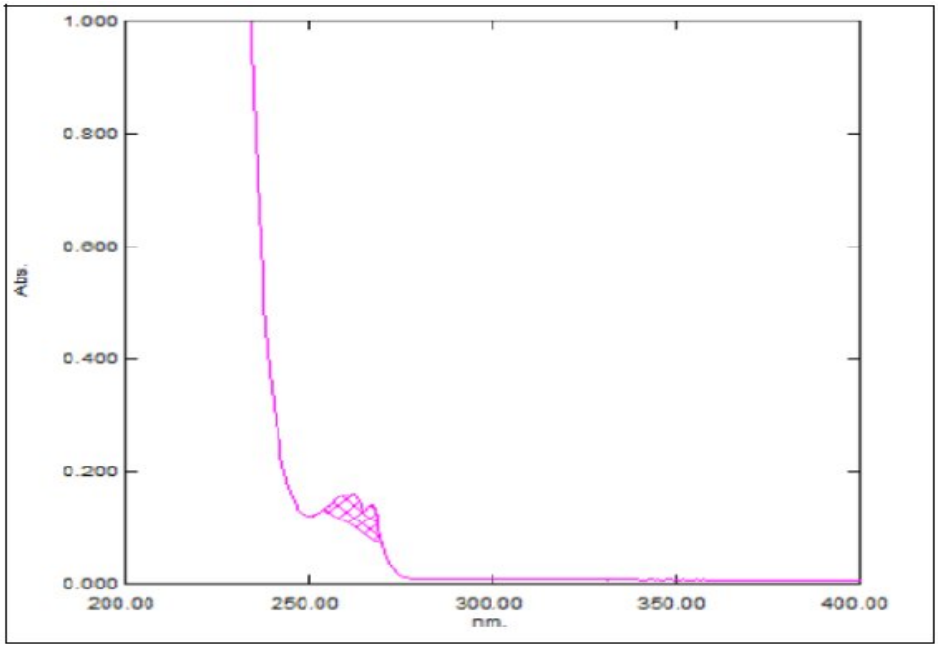


Fig: 5.3 Typical Zero order spectra of Rufinamide showing Area Under Curve [AUC] from 254 to 270 nm.

Table: 5.6

Data of Calibration Curve for Rufinamide by AUC method

Sl. no.	Conc. (mcg / ml)	Absorbance between 254-270 nm
1	100	0.125
2	200	0.265
3	300	0.380
4	400	0.490
5	500	0.616
6	600	0.751

Table: 5.7 Optimum conditions, Optical characteristics and Statistical data of linearity for Rufinamide by AUC method

Parameters	UV Method
Range to measure AUC (nm)	254-270
Beer's law limits (mcg / ml)	100-600
Molar extinction coefficient (L mol^{-1} cm^{-1})	0.00125×10^4
Sandell's sensitivity (mcg/cm^2-0.001 absorbance units)	0.816
Regression equation (Y*)	Y = 0.00123C + 0.00475
Slope (b)	0.00123
Intercept (a)	0.00475
Correlation coefficient(r^2)	0.9996
Intraday Precision (% RSD**) Inter day Precision (% RSD**)	0.166 0.182
Limit of detection (mcg / ml)	1.67
Limit of quantitation (mcg / ml)	5.08

*Y = b X + a where X is the concentration of Rufinamide in µg / ml and Y is the AUC in between 254 nm to 270nm.

#A = Absorbance unit.

**Mean value obtained from 6 linearity curves.

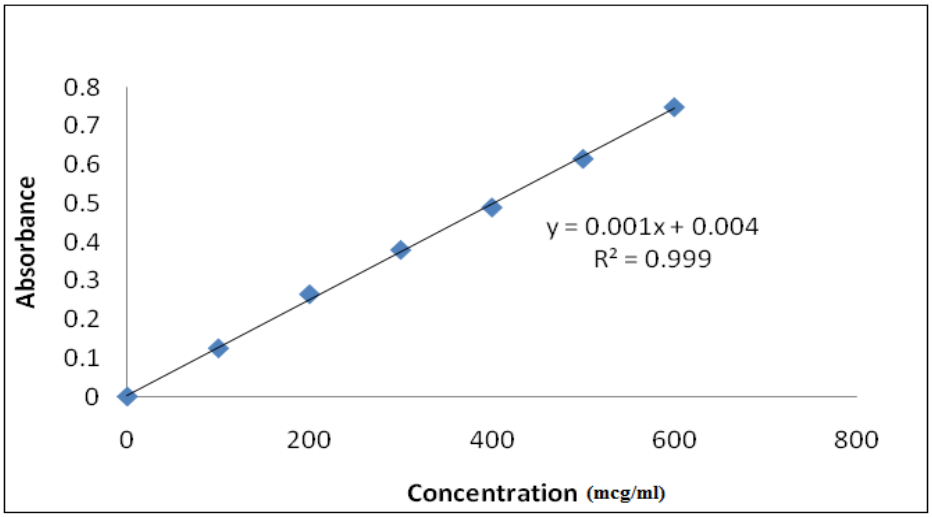


Fig: 5.4 Calibration curve for RUFINAMIDE by AUC method

Table: 5.8

Data of Intraday Precision study for Rufinamide by AUC method

Conc. mcg / ml	Intra-day Absorbance Mean ± SD**	% RSD	Inter-day Absorbance Mean ± SD**	% RSD
100	0.01248 ± 0.000753	0.603022	0.1250 ± 0.001095	0.876356
200	0.26489 ± 0.000753	0.284244	0.2641 ± 0.000894	0.338798
300	0.380167 ± 0.00075	0.198011	0.3802 ± 0.000894	0.2235376
400	0.48933 ± 0.000816	0.166859	0.489 ± 0.008941	0.182909
500	0.6160 ± 0.001265	0.205343	0.61616 ± 0.00116	0.189729
600	0.7501 ± 0.000894	0.119257	0.74966 ± 0.00121	0.161546

*Obtained from 18 determinations (6 determinations per day).

Table: 5.9

Data of Recovery study for Rufinamide by AUC method

Brand used	Amount of sample (µg / ml)	Amount of drug added (µg / ml)	Amount Recovered	% Recovery ± SD**	% RSD
BRAND X	400	200	199.66	101.93±0.45	0.47
	400	400	400.66	99.08±0.48	0.48
	400	600	599.6	100.23±0.17	0.20

Table: 5.10

Data of Ruggedness and Assay for Rufinamide formulations by AUC method

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
BRAND X	400	399.87	99.87 ± 0.48	400.52	100.52 ± 0.204

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In RP-HPLC method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to elute title ingredient. Mobile phase and flow rate selection was based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor) run time and resolution. The system with mobile phase containing Methanol **:** Water (50:50) with 1ml / min flow rate was quite robust. The optimum wavelength for detection was 220 nm at which better detector response for the drug was obtained. For the drug Rufinamide, peak of retention time was 5.203 ± 0.02 min.

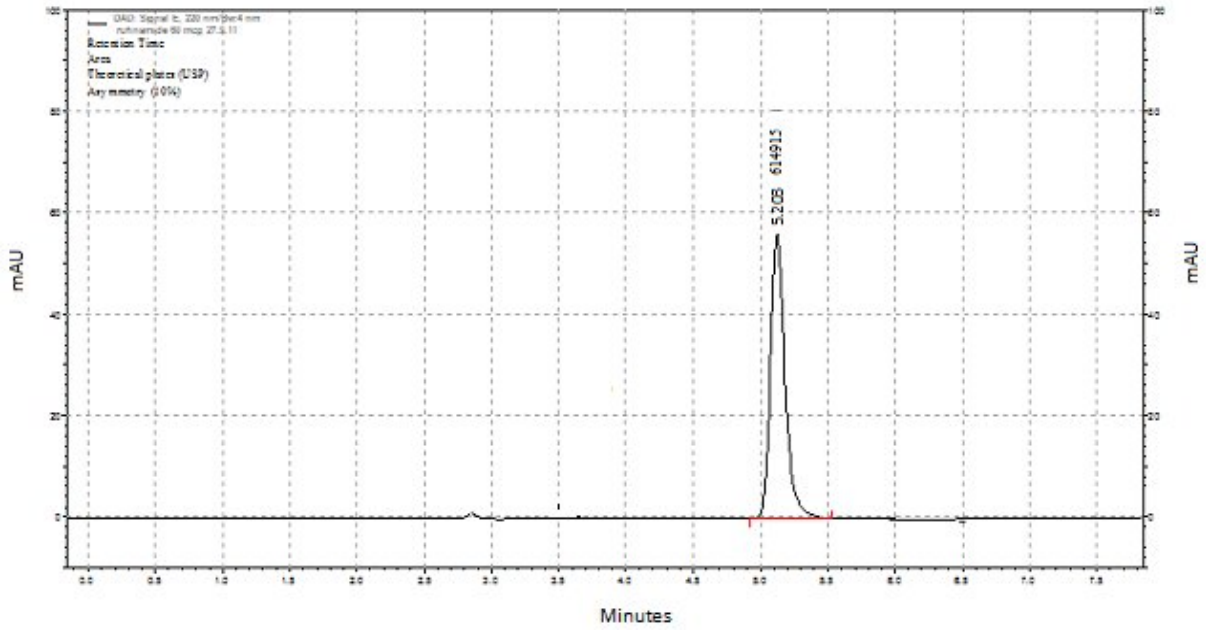


Fig: 5.5. Chromatogram of Rufinamide at 220 nm

Table: 5.11

Characteristic parameters of Rufinamide for the proposed RP-HPLC method

Parameters	RP-HPLC
Calibration range (mcg / ml)	10-60
Detection wavelength	220 nm
Mobile phase (Methanol : Water)	50 : 50 v/v
Retention time	5.203 ± 0.02
Regression equation (Y*)	y = 14860x + 10661
Slope (b)	14860
Intercept (a)	10661
Correlation coefficient(r^2)	0.999
Intraday Precision (% RSD*) Interday Precision (% RSD*)	1.05 1.02
Limit of detection (mcg / ml)	1.51
Limit of quantitation (mcg / ml)	4.60

*Y = b C + a, where X is the concentration of compound in mcg / ml and

Y is the peak area.

VALIDATION OF ANALYTICAL METHOD:

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics were expressed in terms of analytical parameters.

1. ACCURACY:

The accuracy of the method was inferred by establishing the precision and linearity studies of standard drug.

Table: 5.12.

Accuracy results for Rufinamide

Sample No.	Spike Level	Amount (mcg / ml) added	Amount (mcg / ml) found	% Recovery	Mean % Recovery
1	50 %	20	20.10	101.58	100.93
	50 %	20	19.89	100.55	
	50 %	20	20.13	101.40	
2	100 %	40	39.79	98.91	99.08
	100 %	40	40.21	99.44	
	100 %	40	39.75	98.45	
3	150 %	60	60.13	100.37	100.28
	150 %	60	59.89	100.18	
	150 %	60	60.10	100.52	

2. *PRECISION:*

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision results were expressed as standard deviation or relative standard deviation.

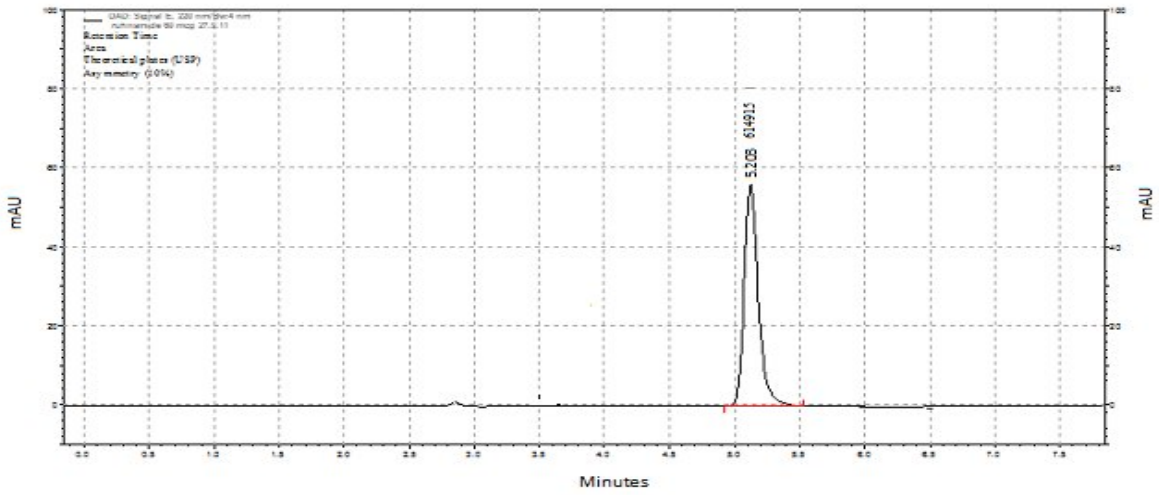


Fig: 5.6. Chromatogram of Rufinamide at 220 nm

Table: 5.13.

Precision results for Rufinamide

Sl. No.	**Concentration (mcg / ml)**	**Intraday precision** (Area)	**Interday precision** (Area)
1	40	610562	609231
2	40	614915	604938
3	40	609231	599231
4	40	599231	609231
5	40	604915	599231
6	40	599234	614915
Mean		606347.5	606129.5
Std.Dev		6368.985	6213.242
%RSD.		1.05	1.02

** Average of Six determinations

3. LINEARITY:

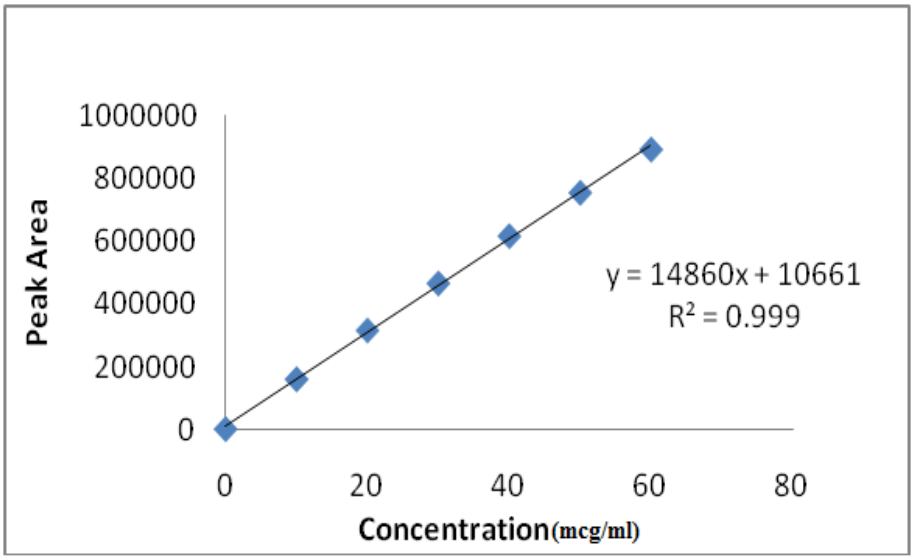


Fig: 5.7. Calibration curve of Rufinamide at 220 nm

Table: 5.14.

Linearity results for Rufinamide

Sl. no.	Conc. (mcg / ml)	Peak Area
1	10	158751
2	20	314117
3	30	464005
4	40	614915
5	50	752760
6	60	890650

** Average of Six determinations.

Table: 5.15.

Calibration parameters of Rufinamide

Parameter	Results
Slope	14860
Intercept	10661
Correlation co-efficient	0.999
% Percentage Curve Fitting	99.9

Table: 5.16.

System suitability studies of Rufinamide by RP-HPLC method

Property	Values	Required limits
Retention time (R_t)	5.203 ± 0.02	RSD ≤ 1%
Theoretical plates (N)	2704	$N > 2000$
Tailing factor (T)	1.5	$T \leq 2$

4. LIMIT OF DETECTION (LOD):

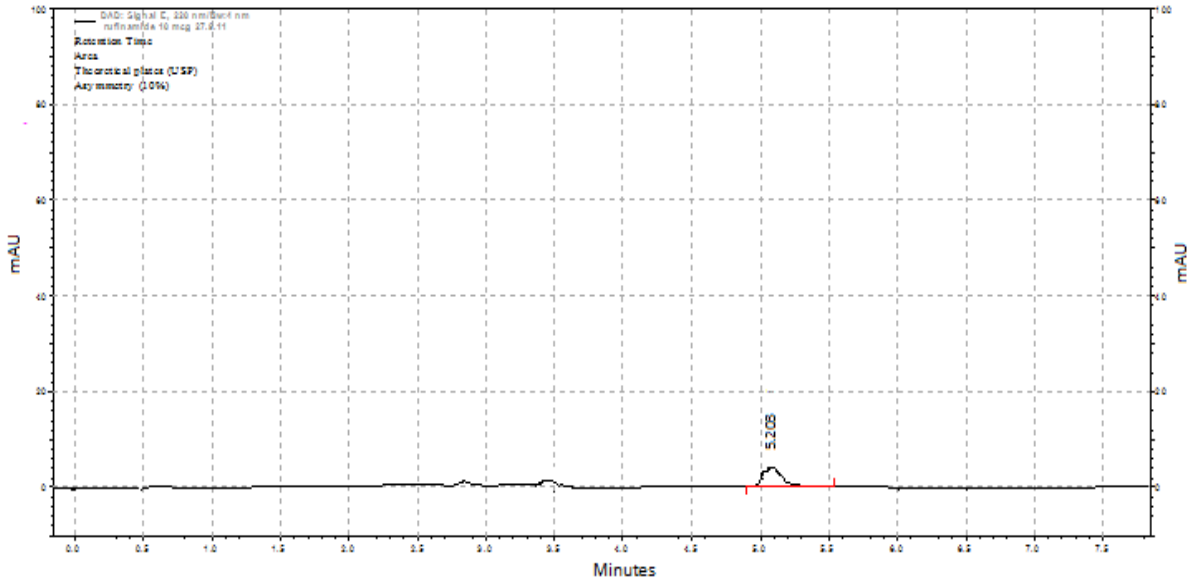


Fig: 5.8. Chromatogram of Limit of detection

Table: 5.17.

LOD results for Rufinamide

Injection No.	Peak Area	% RSD
1	3402	2.82

5. LIMIT OF QUANTITATION (LOQ):

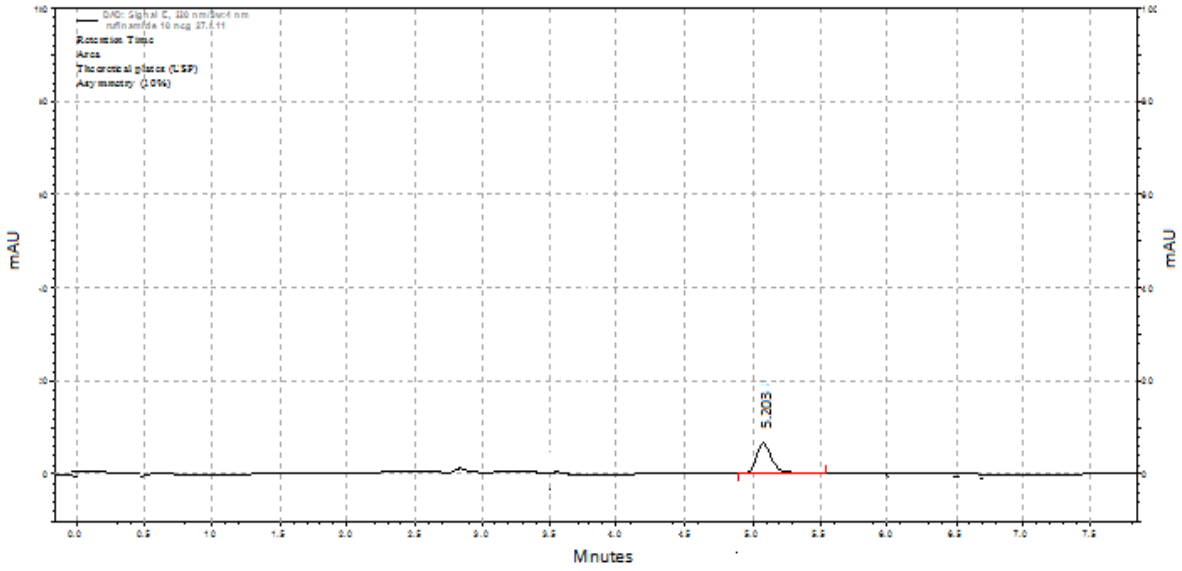


Fig: 5.9. Chromatogram of Limit of Quantitation

Table: 5.18.

LOQ results for Rufinamide

Injection No.	Peak Area	% RSD
1	5739	10

6. RUGGEDNESS:

Ruggedness is a measure of the reproducibility of a test result under normal, expected operating condition from instrument to instrument and from analyst to analyst.

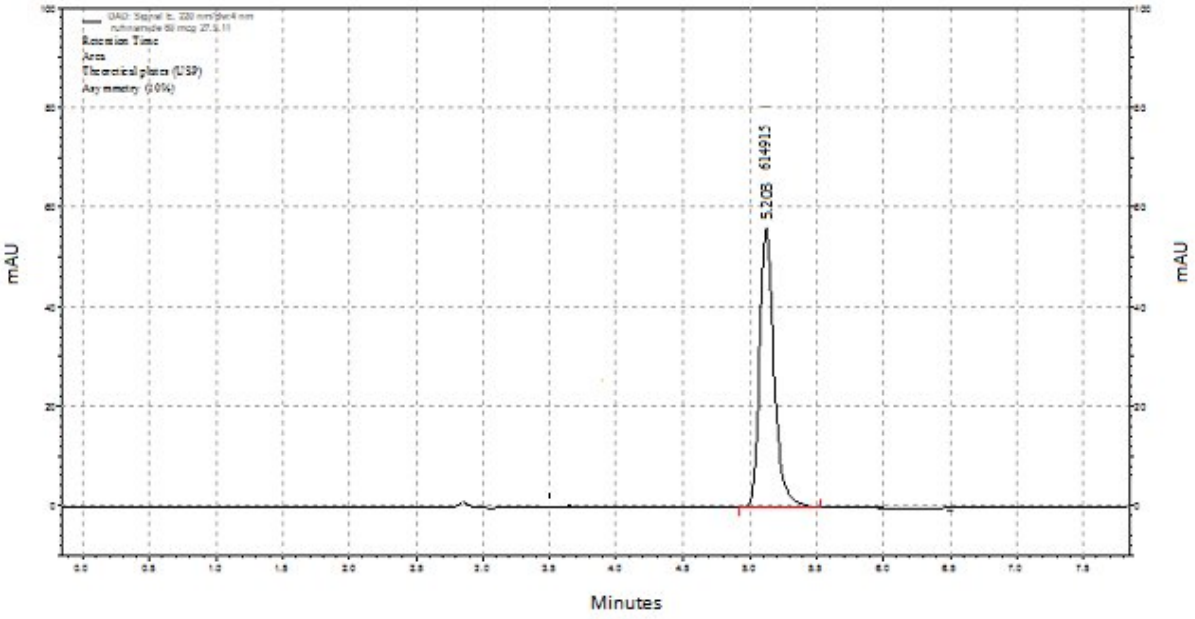


Fig: 5.10. Chromatogram of Ruggedness

Table: 5.19.

Ruggedness studies of Rufinamide by RP-HPLC method

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
Sample	400	399.91	99.08 ± 0.48	400.21	100.28 ± 0.17

7. ROBUSTNESS:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Table: 5.20.

(a). Chromatographic Condition: Change in flow rate

SI. No	Change in flow rate	R.T
01	0.9 ml / min	5.8
02	1.1 ml / min	4.7

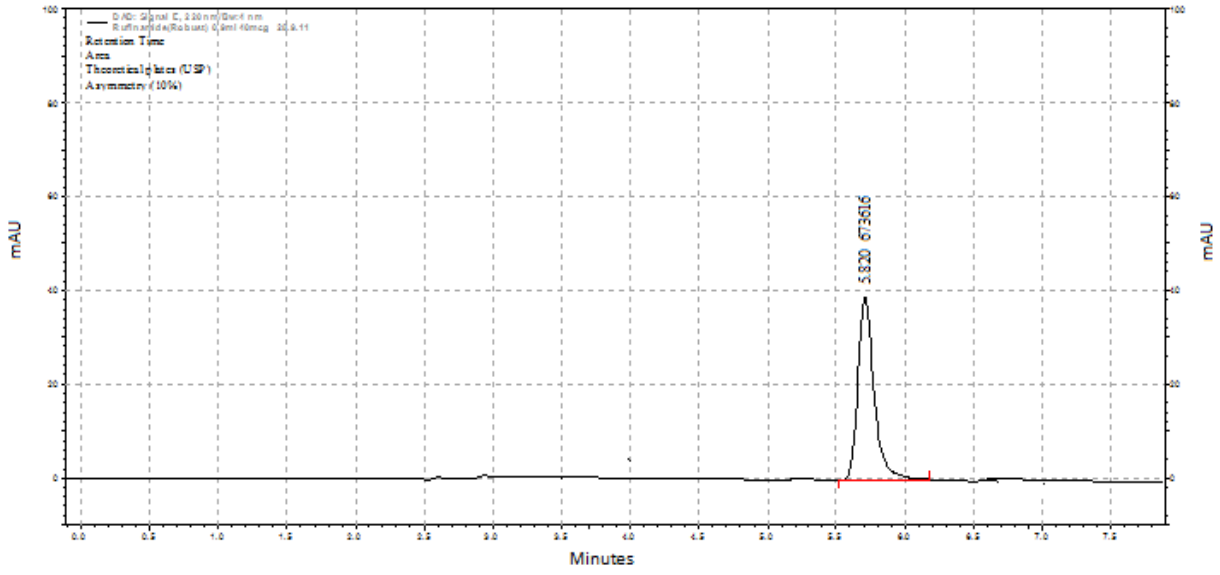


Fig: 5.11. Chromatogram of Robustness (Flow rate 0.9ml)

Table: 5.21.

Robustness results for Rufinamide: (Flow rate 0.9ml)

SL. NO.	RT	Peak Area	USP Plate Count	USP Tailing
1	5.8	606347	3364	1.5

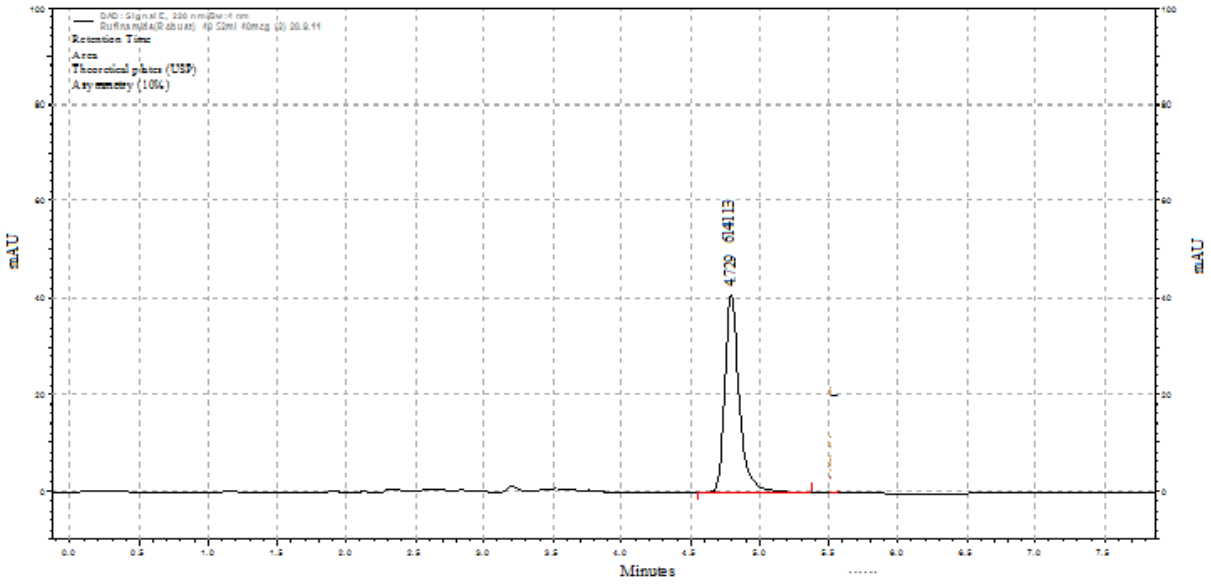


Fig: 5.12. Chromatogram of Robustness (Flow rate 1.1ml)

Table: 5.22.

Robustness results for Rufinamide: (Flow rate 1.1ml)

Sl.NO.	RT	Peak Area	USP Plate Count	USP Tailing
1	4.7	528853	2209	1.5

Table: 5.23.

(b). Chromatographic Condition: Change in mobile phase

SI. No	Change in mobile phase	R.T
01	48 : 52	4.92
02	52 : 48	5.66

Mean ± S.D. from six determinations.

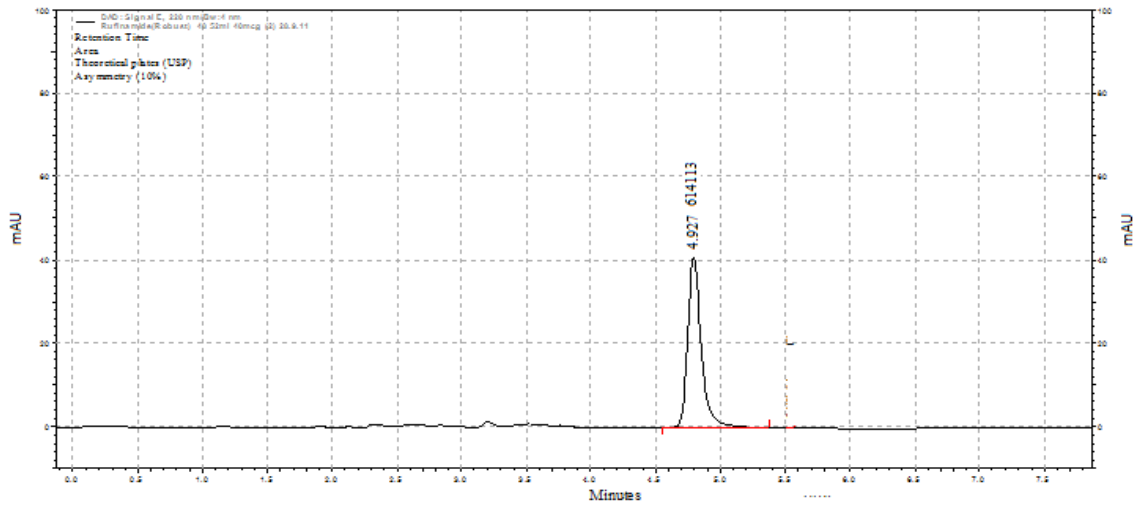


Fig: 5.13. Chromatogram of Robustness (Water: Methanol = 48:52)

Table: 5.24.

Robustness results for Rufinamide: (Water: Methanol= 48:52)

SL.NO.	RT	Peak Area	USP Plate Count	USP Tailing
1	4.92	649449	2420.6	1.45

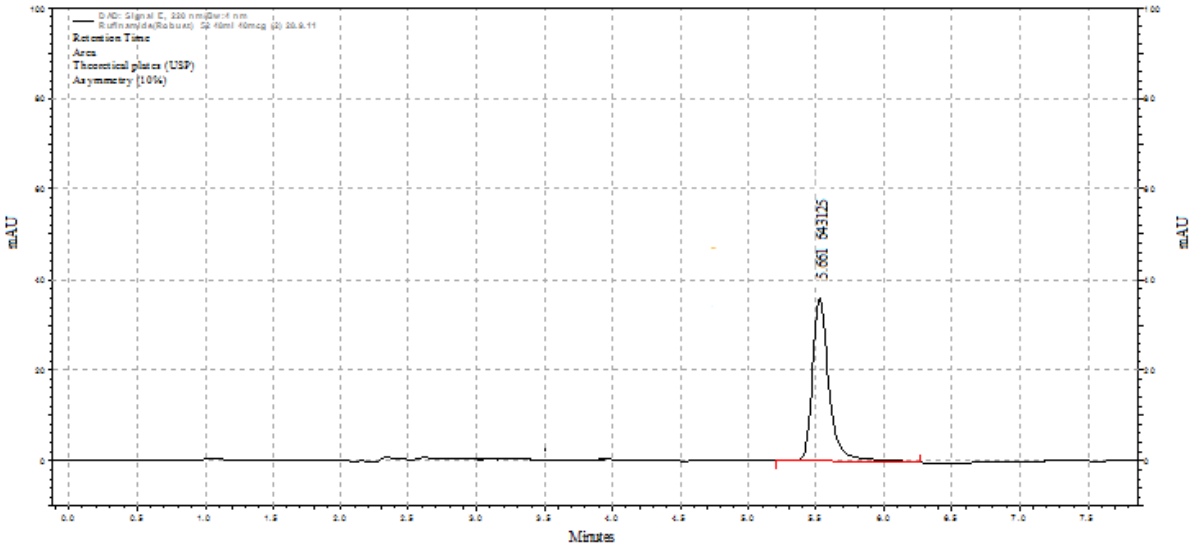


Fig: 5.14. Chromatogram of Robustness (Water: Methanol= 52:48)

Table: 5.25.

Robustness results for Rufinamide: (Water: Methanol= 52:48)

Sl.NO	RT	Peak Area	USP Plate Count	USP Tailing
1	5.66	519380	3203.5	1.5

Table: 5.26.

(b). Chromatographic Condition: Change in Wavelength

Sl. No	Change in Wavelength	R.T
01	217	5.2
02	223	5.2

Mean ± S.D. from six determinations.

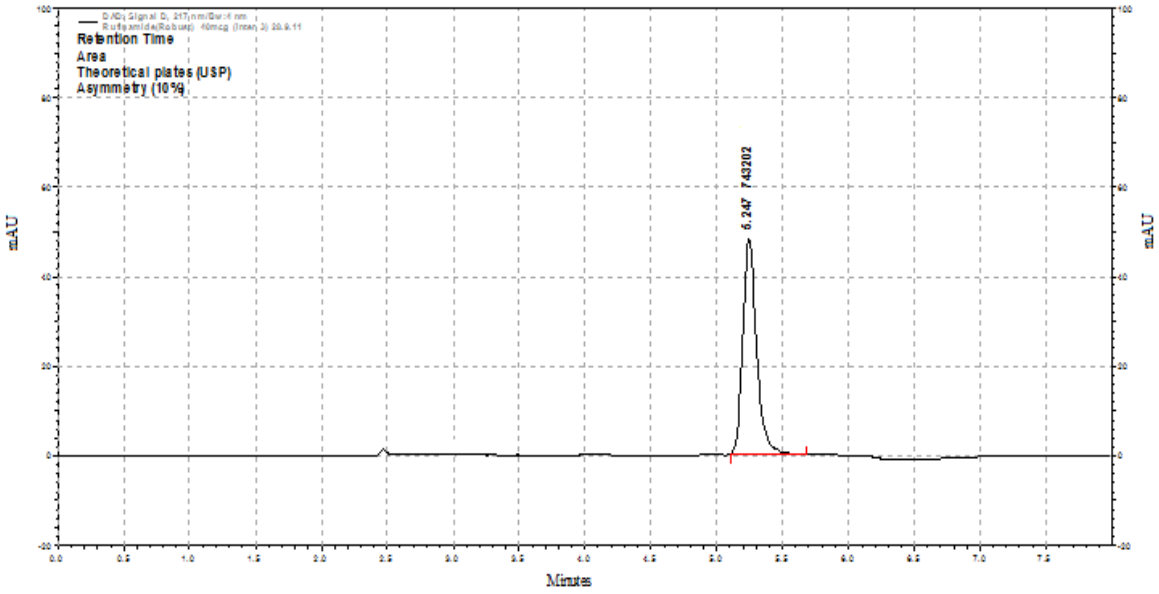


Fig: 5.15. Chromatogram of Robustness (Wavelength 217nm)

Table: 5.27.

Robustness results for Rufinamide (Wavelength 217nm)

Sl. NO.	RT	Peak Area	USP Plate Count	USP Tailing
1	5.2	743202	2704	1.5

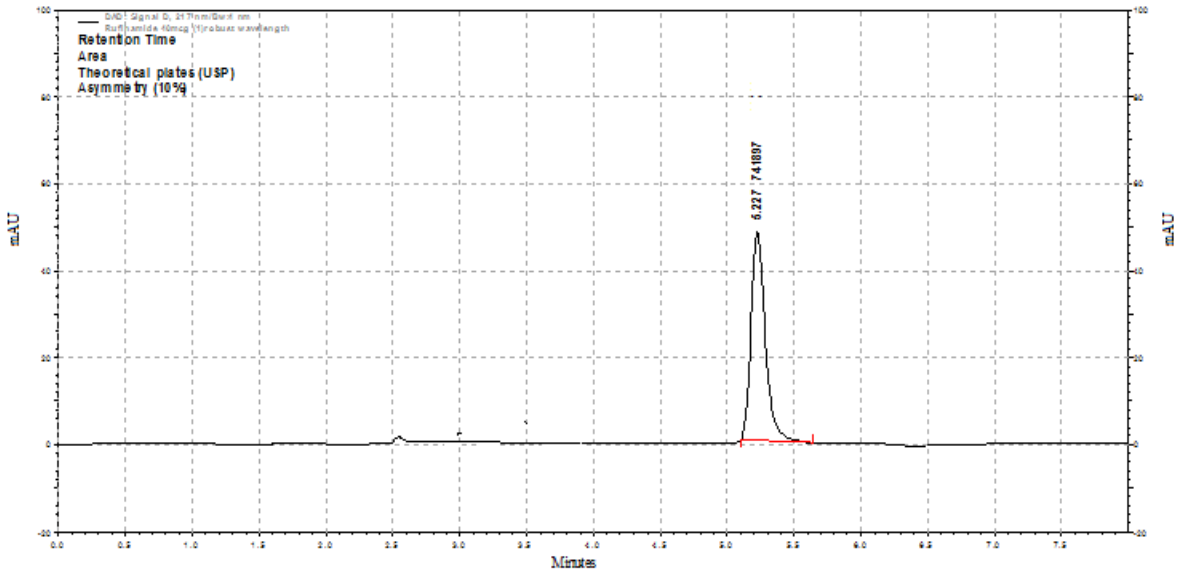


Fig: 5.16. Chromatogram of Robustness (Wavelength 223nm)

Table: 5.28.

Robustness results for Rufinamide (Wavelength 223nm)

Sl. NO.	RT	Peak Area	USP Plate Count	USP Tailing
1	5.2	741897	2704	1.5

6. DISCUSSION

All the proposed methods was found to be simple, rapid, accurate and reproducible for the determination of Rufinamide and methods was validated according to USP and ICH guidelines for different parameters.

PART A: UV SPECTROSCOPY

The spectrophotometric methods developed were:

Method A: Zero Order Derivative Spectroscopy

Method B: AREA UNDER THE CURVE METHOD

After considering the solubility and stability, DMSO and distilled water was selected as the common solvent. The absorption spectra were recorded in the wavelength region of 200-400 nm in UV method. The spectra are presented as **Fig: 5.1 and 5.3.**

Beer's law range was confirmed by the linearity of the calibration curve of Rufinamide which were represented in **Fig: 5.2 and 5.4.** Rufinamide showed linearity in the concentration range of 100-600 mcg / ml in Zero order and AUC method derivative Spectroscopy, respectively.

The optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity, slope (b), intercept (C), Sandell's sensitivity, correlation coefficient (r^2) obtained from different concentrations, percent relative standard deviation, LOD and LOQ values were presented in **Table: 5.2 and 5.7**. The results showed that these methods have reasonable precision.

Results obtained with proposed methods confirm the suitability of these methods for pharmaceutical dosage forms. The accuracy of the methods were confirmed by the recovery studies, by adding known amount of the pure drug to the pharmaceutical

formulation and the percentage recovery studies were determined and data were presented in analytical **Tables: 5.3 and 5.9,** respectively. The results were within the range of 99.08 -101.93 % and were found to be highly accurate.

Ruggedness test expresses the precision of the method. The ruggedness results were shown in **Table: 5.5 and 5.10,** respectively. The results were found to be highly precise.

The other active ingredients and common excipients present in the dosage forms of Rufinamide did not interfere, when added in the mentioned concentration ranges to the drug and estimated by the proposed methods. The methods reported here are found to be simple, sensitive, accurate, precise, and economical can be used in the determination of Rufinamide from pharmaceutical formulations in a routine manner.

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In HPLC method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds. The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of Rufinamide in bulk drug and pharmaceutical dosage form by using the most commonly employed Qualisil gold C-18 column with DAD or PDA detector.

The run time was set at 8 min and the retention time for Rufinamide was 5.20 ± 0.02 min. Each sample was injected 5 times and the retention times were same. When the concentrations of Rufinamide and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship (r^2= 0.999) was observed between the concentration of Rufinamide and the respective peak areas in the range 10-60 mcg / ml. The regression equation was used to estimate the amount of Rufinamide, either in tablet formulations or in validation study (precision and

accuracy). For the proposed RP-HPLC method, characteristic parameters were shown in **Table: 5.11.**

To analyse tablet formulations, RP-HPLC method has been developed. Rufinamide tablets were analyzed as per the procedure described above. The low % RSD values (≤ 2) indicated that the method was precise and accurate. The mean recoveries were found in the range of 99.08 – 100.93 %. No interfering peaks were found in the chromatogram indicating that excipients used in the tablet formulation did not interfere with the estimation of the drug by the proposed RP-HPLC method.

The proposed RP-HPLC method was also validated for intra and inter-day variation. When the solution containing 40 mcg/ml of Rufinamide was repeatedly injected on the same day, the %RSD in the peak area for six replicate injections was found to be 1.05% Also the inter day variation (6 days and six injections) was found to be 1.02%. The results are presented in **Tables: 5.13.** The % RSD values were within 2 and the method was found to be precise.

Keeping the flow rate constant (1 ml / min), the chromatograms of drug solution were recorded by changing mobile phase ratios such as Water: Methanol = 50:50, 52:48 and 48:52(v/v). With the mobile phase Water: Methanol (50:50, v / v,), the peaks were sharp with good resolution. The results are presented in **Table 5.23**. These values indicated that the method was quite robust.

Keeping the ratio of mobile phase constant Water: Methanol (50:50, v / v,), the chromatograms of drug solution were recorded with different flow rates such as 0.9 ml / min, 1 ml / min and 1.1 ml / min. With the flow rate of 1 ml / min, the peaks were sharp with good resolution. The results were presented in **Table: 5.20.**

Keeping the ratio of flow rate (1 ml / min) and mobile phase constant Water: Methanol (50:50, v / v,), the chromatograms of drug solution were recorded with different wavelengths such as 217 nm, 220 nm and 223 nm. With the wavelength of 220 nm, the peaks were sharp with good resolution but with other wavelength results were not satisfactory. So 220 nm is kept constant for the analysis. The results were presented in **Table: 5.26.**

The assay of Rufinamide was performed by different analyst and on different dates (days). The % assay was calculated and those values were given in **Table: 5.19**. The results were reported to be within the limits.

7. CONCLUSION

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the three methods were complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of Rufinamide in bulk and pharmaceutical dosage forms.

A very few analytical methods appeared in the literature for the determination of Rufinamide, which includes HPLC, UV-Vis Spectrophotometric methods and LC-MS / MS methods has been reported for Rufinamide. In view of the above fact, some simple analytical methods were planned to develop with sensitivity, accuracy, precision and economical.

In Part A, two simple and sensitive UV/Visible spectrophotometric methods for the quantitative estimation of Rufinamide in bulk drug and pharmaceutical formulations have been developed.

In order to ensure that the data generated in each of the above methods were accurate and precise. The results were expressed in **Table: 5.1 - 5.10** for Spectrophotometric methods. The low value of % RSD and values of Molar extinction coefficient (mol^{-1} cm^{-1}) and Sandell's sensitivity (mcg/cm^2) suggested that the developed method may be sensitive. Recovery studies indicate that there is no interference due to excipients.

In addition to positive requirements for analytical methods, the striking advantage of all the presently developed methods is that they are economical.

These methods are validated in terms of accuracy, precision, repeatability, ruggedness and can be used for the routine determination of Rufinamide in bulk drug and pharmaceutical formulations.

In Part B the present investigation, simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Rufinamide in its bulk and pharmaceutical dosage forms. The results are expressed in **Table: 5.11 – 5.28**.

The RP-HPLC method was more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine determination of Rufinamide in bulk drug and in pharmaceutical dosage forms.

8. SUMMARY

In the present work, an attempt was made to provide a newer, simple, accurate and low cost spectrophotometric methods and one HPLC method for the effective quantitative determination of Rufinamide as an active pharmaceutical ingredient as well as in pharmaceutical preparations without the interferences of other constituent in the formulations.

For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision. Spectrophotometric technique, derivative method were applied without using any prior chemical pretreatment in the presence of the strongly overlapping spectra can generate large amounts of data within a short period of analysis.

Pharmaceutical analysis occupies a pivotal role in statutory certification of drugs and their formulations either by the industry or by the regulatory authorities. There is a wide scope for the development of new analytical methods for the assay of the above drug. Among several instrumental techniques (HPLC, GLC, Fluorimetry, IR, UV, Visble, NMR, and Mass spectrometry) available for the assay of drugs. HPLC technique has been regarded as the best among various instrumental ones in spite of its sensitivity and maintenance problems.

UV Spectrophotometry and RP-HPLC techniques have been used as tools in the present work. The above tools have been used for the development of new analytical methods for the assay of Rufinamide. The contents of the thesis have been divided into nine chapters.

Chapter 1 begins with the introduction giving a brief account of pharmaceutical analysis, analytical method development, UV-Spectrophotometry, Zero order Spectrophotometric method, AUC method, High Performance Liquid Chromatography,

and analytical method validation. It also includes drug profile of Rufinamide with physicochemical characteristics and specifications for the drug.

Chapter 2 explains the objectives of present investigations adopted for selected drug.

Chapter 3 surfaces review of literature that gives important details collected from previously published analytical methods for Rufinamide.

Chapter 4 presents methodology which is divided into two parts, Part A and Part B. In Part A, elaborated methodologies for the proposed two different UV-Spectrophotometric methods are included while in Part B, detailed methodologies for a RP-HPLC method is included.

Chapter 5 consists of results which are divided into two parts, Part A and Part B. In Part A, the results obtained in Zero order and AUC UV-Spectrophotometric methods for determination of Rufinamide are given in their respective tables. In Part B results obtained from RP-HPLC method for estimation of Rufinamide are illustrated in their respective tables.

Chapter 6 consists of discussion which discusses about the developed UV spectrophotometric methods and RP-HPLC method.

Chapter 7 explains the conclusion in two parts, Part A and Part B respectively. Part A includes the proposed UV spectrophotometric methods for the quantitative estimation of Rufinamide. Part B contains proposed RP-HPLC method for the quantitative estimation of Rufinamide. These methods are validated in terms of sensitivity, accuracy and precision and can be used for the routine determination of Rufinamide in bulk drug and pharmaceutical formulations.

Chapter 8 contains the summary.

Chapter 9 contains the bibliography.

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